MECHANISM AND FUNCTIONAL SIGNIFICANCE OF GLT-1 DOWNREGULATION BY COCAINE

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ABSTRACT

Ronald Kim: Mechanism and Functional Significance of
GLT-1 Downregulation by Cocaine
(Under the direction of Kathryn J. Reissner)

Neurobiological adaptations in the nucleus accumbens (NAc) following withdrawal from drug use are associated with vulnerability to relapse. Among the identified changes in the NAc is decreased expression of the glutamate transporter GLT-1. Because GLT-1 is primarily expressed on astrocytes, an important and related question emerges regarding what other adaptations occur in astrocytes subsequent to drug use. Recent studies indicate that short-access cocaine self-administration and extinction training in rats leads to a decrease in the surface area, volume, and synaptic colocalization of NAc astrocytes. However, it is currently unknown if these findings extend to other rodent models of cocaine self-administration. Moreover, it is unknown whether these findings extend to female rats. Therefore, the goals of the dissertation were to examine the effects of long-access (LgA) cocaine self-administration and prolonged abstinence on (1) NAc GLT-1 expression in female rats and (2) the structural plasticity of NAc astrocytes in both sexes. Finally, a third goal of this dissertation was to examine if changes in NAc GLT-1 expression and NAc astrocytes are correlational or mechanistically linked.

The experiments described in Chapter 2 revealed that GLT-1 mRNA and protein levels are unchanged in the NAc of female rats, despite the fact that both are decreased in males. The experiments described in Chapter 3 showed that although astrocytes from male rats exhibited a decrease in surface area, volume, and synaptic colocalization, these changes were not

observed in female rats. Finally, the experiments described in Chapter 4 showed that although GLT-1 overexpression in the NAc had no effect on the cocaine-induced decrease in the surface area and volume of astrocytes, it significantly increased synaptic colocalization. The results from Chapter 4 also showed that NAc GLT-1 overexpression had no effect on the incubation of cocaine craving. Collectively, these data suggest sex differences in GLT-1 expression, as well as in the changes in NAc astrocytes following LgA cocaine self-administration and abstinence. These results also show that the downregulation in GLT-1 expression and the cocaine-induced changes in NAc astrocytes are not mechanistically linked, and further, that GLT-1 overexpression alone is not sufficient to attenuate cocaine seeking.

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AAV	Adeno-associated virus
Akt	Protein kinase B
ALDH1L1	Aldehyde dehydrogenase 1 L1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
cDNA	Complementary deoxyribonucleic acid
Cx30	Connexin 30
DA	Dopamine
DAT	Dopamine transporter
DREADD	Designer receptors exclusively activated by designer drugs
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
FR1	Fixed ratio 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter type I
GluA1	Glutamate ionotropic receptor AMPA subunit type 1

GluA2	Glutamate ionotropic receptor AMPA subunit type 2
HA	Hemagglutinin
HCl	Hydrochloric acid
IV	Intravenous
i.p.	Intraperitoneal
i.v.	Intravenous
iGluR	Ionotropic glutamate receptor
Lck	Lymphocyte protein tyrosine kinase
LgA	Long-access
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NAC	N-acetylcysteine
NGS	Normal goat serum
NIDA	National Institute of Drug Abuse
NMDA	N-methyl-D-aspartate
MAPK	Mitogen-activated protein kinase
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + triton x-100
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PJNK	Phospho-jun amino terminal kinase

PPF	Propentofylline
PSD 95	Post synaptic density 95
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
Rac1	Ras related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation
ROCK	Rho kinase
RNA	Ribonucleic acid
ROI	Region of interest
SA	Self-administration
SDS	Sodium dodecyl sulfate
ShA	Short-access
SUD	Substance use disorder
TBS	Tris buffered saline
TNFα	Tumor necrosis factor alpha
xCT	Catalytic subunit of the cystine-glutamate exchanger

Chapter 1

GENERAL INTRODUCTION

Substance Use Disorder

Drug addiction is a devastating chronic disorder characterized by both physiological and psychological symptoms. The Diagnostic and Statistical Manual of Mental Disorders (DSM-V) defines diagnostic criteria for substance use disorder (SUD) which include: the development of drug tolerance, the onset of withdrawal symptoms, the inability to quit using drugs despite the desire to do so, and the continued use of drugs despite negative social and economic consequences (Liu and Li, 2018). Recent statistics indicate that as of 2017, as many as 19.7 million people in the United States (approximately 7.2% of the population) meet DSM-V criteria for SUD (Uhl et al., 2019). Of these, roughly 7.5 million people are estimated to use illicit drugs which include marijuana, opioids, and stimulant drugs such as amphetamine and cocaine. Furthermore, the number of deaths related to drug overdose reached 70,237 in 2017 (National Institute on Drug Abuse (NIDA), CDC Wonder). This number is approximately double the number of drug related overdoses from 2007, and almost quadruple the number from 1999 (NIDA, CDC Wonder). SUD also leads to a tremendous economic burden on society. NIDA estimates that approximately $700 billion is spent annually due to lost work productivity related to drug use, the cost of law enforcement associated with drug enforcement, and to treat health problems associated with drug use (NIDA).

To combat the myriad of problems associated with drug addiction, better treatment options for SUD are needed. Behavioral therapies such as cognitive behavior therapy, mindfulness training, and cue exposure therapy, have all shown some promise as non-pharmacological treatment options for SUD (Zilverstand et al., 2016). However, the effectiveness of these behavioral therapies is often temporary (Liu and Li, 2018) and do not address the underlying neurobiological changes associated with drug use. In addition, the high economic cost associated with such therapies makes finding alternative treatments for SUD paramount (Carroll, 2014). Therefore, pharmacotherapeutic treatments represent an alternative treatment option for SUD. To date, a handful of pharmacological treatment options have been approved by the Food and Drug Administration (FDA) to target SUD. For example, the opioid receptor antagonist naltrexone is used to reverse overdose to heroin and other opioids (Larney et al., 2014), naltrexone, acamprosate and disulfiram have been approved to treat alcohol dependence (Warren, 2019), and bupropion and varenicline have been FDA approved to target nicotine dependence (Mills et al., 2012).

Despite these advances in pharmacotherapeutic treatment options for alcohol and nicotine dependence, there are currently no FDA approved pharmacological treatments for dependence to stimulants such as cocaine and amphetamine. Furthermore, although these FDA approved pharmacological treatments have been moderately effective in reducing craving and easing withdrawal symptoms, they have not been as effective in preventing relapse (Liu and Li, 2018). Relapse is one of the hallmark symptoms of drug addiction and SUD, and is defined as the return to drug use following a period of abstinence. Current estimates indicate that approximately two-thirds of drug users who wish to quit using drugs relapse within a few months of seeking treatment, and an overwhelming 85% of users ultimately relapse within one

year (Sinha, 2011). These staggering statistics highlight the importance of continued research into developing effective pharmacotherapeutic treatment options for relapse.

The drug self-administration paradigm as a rodent model of relapse

Animal models of drug addiction have proven to be critical in identifying neural substrates of drug reward, dependence and seeking, and thus, provide an important resource for developing candidate pharmacological treatments for drug addiction. Over the past several decades, multiple different preclinical animal models have been developed to examine various aspects of addiction. For example, behavioral sensitization to psychostimulants is assessed by measuring increased locomotor responsiveness across multiple drug exposures (Dafny and Yang, 2006), while the subjective effects of drugs of abuse have been observed using the drug discrimination paradigm (Jaramillo et al., 2017), and the motivational properties of drugs have been examined using the conditioned place preference paradigm (Tzschentke, 2007). However, in the majority of these models, the drug of choice is often non-contingently administered by the experimenter, thus making it difficult to emulate human drug addiction. Therefore, in animal models of drug abuse, the drug self-administration paradigm has emerged as the hallmark for modeling multiple aspects of drug addiction, including relapse (Shaham et al., 2003).

In the drug self-administration paradigm, administration of the drug is contingent on the ability of the animal to perform a behavior such as a lever press or a nose poke (Roberts et al., 2007). This operant conditioning paradigm builds off the work done by B.F. Skinner and is an example of positive reinforcement. The drug of abuse acts as the reinforcer and due to the rewarding and reinforcing effects of these drugs, an increase in the behavioral response for the drug (i.e. a lever press or nose poke) is observed. Furthermore, depending on the drug of

interest, the route of drug administration can be altered. For drugs such as alcohol which are typically orally administered, operant behavior can result in the delivery of alcohol which can be orally administered by the animal (Samson et al., 1988). In contrast, for stimulant drugs such as cocaine and amphetamine, intravenous (i.v.) drug delivery has proven to be most efficient (Roberts et al., 2007; Shaham et al., 2003). In this case, animals are implanted with a jugular vein catheter and operant behavior results in an i.v. infusion of drugs. The rapid pharmacodynamics of i.v. drug self-administration has proven to be effective in modeling human drug intake behavior in rodents (Gardner, 2000; Koob and Weiss, 1990).

In a typical i.v. drug self-administration experiment, following recovery from surgical procedures, animals are placed in operant conditioning chambers where a lever press results in an i.v. infusion of a drug such as cocaine. The operant chambers are normally equipped with the ability to present audio and visual cues contingent upon a lever press, which can in turn serve as discrete stimuli and secondary reinforcers (Roberts et al., 2007). For example, in a typical cocaine self-administration paradigm, a lever press on the "active" lever will result in the delivery of cocaine, along with the presentation of both an audio cue (a 5-sec tone) and a visual cue (a light turning on for 5-sec) (Kim et al., 2018b; Sepulveda-Orengo et al., 2018). In contrast, lever presses on the "inactive" lever will not result in an infusion of cocaine, nor the presentation of any cocaine-related audio or visual cues. Experiments have generally used a moderate dose of cocaine (0.2 mg per infusion or 0.5 mg/kg/infusion), where animals can self-administer cocaine for a limited amount of time (i.e. short-access (ShA) cocaine self-administration, 2 hours/day) for approximately two weeks. This procedure results in a clear preference for the "active" lever, and animals demonstrate a stable increase in active lever

presses throughout the duration of self-administration (Kim et al., 2018b; Sepulveda-Orengo et al., 2018).

Following the acquisition of cocaine self-administration, relapse can be modeled in several different ways. To date, the most commonly used method has been the extinction/reinstatement model (Belin-Rauscent et al., 2016; Bossert et al., 2005; Epstein et al., 2006). In this model, previously acquired cocaine self-administration behavior is first extinguished, and then drug seeking is measured in a reinstatement test. Following acquisition of cocaine self-administration, animals undergo extinction training where lever presses on the active lever no longer result in the delivery of cocaine or the presentation of cocaine-related cues (Kim et al., 2018b; Sepulveda-Orengo et al., 2018). Extinction training lasts for approximately 14-16 days, or until animals meet a previously defined extinction criterion (i.e. > 10 active lever presses for two consecutive days) (Kim et al., 2018b; Scofield et al., 2015). Upon the completion of extinction training, drug seeking behavior can be measured in a reinstatement test. Animals can either be administered a bolus dose of cocaine before being placed in the operant conditioning chamber (cocaine-primed), or placed in the operant chamber and reintroduced to cocaine-related cues upon an active lever press (cue-primed). Use of the extinction/reinstatement model has been instrumental in studying the motivational factors that contribute to drug seeking, as well as the underlying neurobiological mechanisms associated with relapse (Dong et al., 2017; Gardner, 2000).

Although the extinction/reinstatement paradigm has proven to be a reliable model to study relapse, its validity in modeling relapse in humans has been questioned (Belin-Rauscent et al., 2016; Sanchis-Segura and Spanagel, 2006). One important factor is that in humans, drug-taking behavior is not extinguished and addicts often go through a period of abstinence

following cessation of drug-taking behavior (i.e. via time spent in rehab clinics or incarceration). Additionally, the extinction/reinstatement model fails to recapitulate many of the physiological symptoms outlined in the DSM-V criteria for SUD such as drug craving, tolerance, and withdrawal. Therefore, an alternative model of i.v. drug self-administration has emerged, which has been postulated to better model drug addiction in humans (Li et al., 2015; Lu et al., 2004; Pickens et al., 2011). In this paradigm, animals typically self-administer higher doses of cocaine (0.75 mg/kg/infusion) for an extended period of time (long-access (LgA) cocaine self-administration, 6 hours per day), for approximately 10-12 days. Unlike the extinction/reinstatement model where animals exhibit stable lever pressing for cocaine, animals exposed to the LgA cocaine self-administration paradigm show an escalation of cocaine-intake (i.e. an increase in the number of cocaine infusions received throughout the duration of self-administration), which is hypothesized to reflect increases in drug craving and the development of tolerance (Ahmed and Koob, 1998; Ferrario et al., 2005). Furthermore, as an alternative to extinction training, animals go through an extended period of abstinence (approximately 30-45 days) in the home cage.

Following abstinence, cue-induced drug seeking can be measured in an extinction test (as opposed to a reinstatement test since animals never extinguished drug-taking behavior). Compared to drug seeking following extinction training, cue-induced drug seeking is significantly higher following abstinence, and significantly increased following LgA vs. ShA cocaine self-administration (Ferrario et al., 2005; Lu et al., 2004). Interestingly, this increase in drug seeking is further augmented with longer abstinence periods, with the highest amount of drug seeking behavior exhibited at ~45 days of abstinence, before beginning to decline after 60 days (Lu et al., 2004; Pickens et al., 2011). This increase in drug seeking following

abstinence from LgA cocaine self-administration has been termed the "incubation of cocaine craving". The incubation of cocaine craving model has been shown to lead to neurobiological changes unseen in the extinction/reinstatement model (Fischer-Smith et al., 2012; Fischer et al., 2013; Kim et al., 2018b; Loweth et al., 2014; Wolf, 2016), which may better resemble the neurobiological changes observed in human drug addicts. Importantly, the incubation of drug craving has also been observed in human drug addicts (Bedi et al., 2011; Parvaz et al., 2016; Wang et al., 2013), indicating the translational relevance of this model.

Neurobiological adaptations in the NAc following cocaine self-administration

Exposure to drugs of abuse such as cocaine leads to numerous changes within the brain, many of which persist following cessation of drug use. The most well characterized of these changes occur in the nucleus accumbens (NAc). The NAc is a brain region in the basal forebrain that integrates projections from limbic brain regions and outputs to motor brain regions, and thus, plays an integral role in many aspects of motivated behaviors related to drug addiction (Mogenson et al., 1980; Roitman et al., 2005). The acute effects of drugs of abuse are dependent in part on elevated catecholamine levels including dopamine (DA) and norepinephrine (NE) in the NAc (Cornish and Kalivas, 2001; Fowler et al., 2001; Volkow et al., 1997). However, the long-term effects of cocaine abuse, including the neurobiological changes associated with relapse, as well as the neuroadaptations that occur during abstinence, have been hypothesized to be due to other neurobiological mechanisms within the NAc.

Thus, while an increase in DA levels is thought to contribute to drug-taking behavior and the acquisition of cocaine self-administration, changes in glutamatergic signaling in the NAc have been shown to underlie drug seeking and relapse (Cornish and Kalivas, 2001; Kalivas, 2009; Kalivas and McFarland, 2003; Kalivas et al., 2003). For example, one

preclinical study measured extracellular levels of both DA and glutamate in the NAc during a cocaine-primed reinstatement test in rats that previously self-administered cocaine, or were given yoked cocaine or saline (McFarland et al., 2003). Predictably, all three groups showed similar levels of basal DA in the NAc and as expected, when administered a bolus dose of cocaine, all three groups showed a significant elevation of released DA levels (McFarland et al., 2003). However, rats that were previously exposed to cocaine (whether self-administered or yoked) showed a decrease in basal glutamate levels in the NAc (McFarland et al., 2003). Moreover, when a bolus dose of cocaine was administered during the reinstatement test, only the rats that had previously self-administered cocaine exhibited a significant increase in extracellular glutamate levels. As expected, this was the only group that demonstrated cocaine-seeking behavior during a reinstatement test (McFarland et al., 2003). The results from this study indicate a direct relationship between glutamate release in the NAc and drug seeking behavior. Moreover, these results highlight the importance of maintaining proper synaptic glutamate concentrations in the NAc during abstinence from cocaine self-administration, and indicate that aberrations in glutamate concentrations may ultimately lead to relapse.

Alterations in glutamate homeostasis in the NAc following cocaine self-administration

Maintenance of homeostatic concentrations of glutamate is achieved via the regulation of glutamate levels within the synaptic and extrasynaptic space (Figure 1.1). This balance of glutamate levels between the two has been termed “glutamate homeostasis”. Glutamate homeostasis is sustained largely through the action of glial cells, which play an important role in the release of glutamate in the extrasynaptic space, as well a vital role in the uptake of

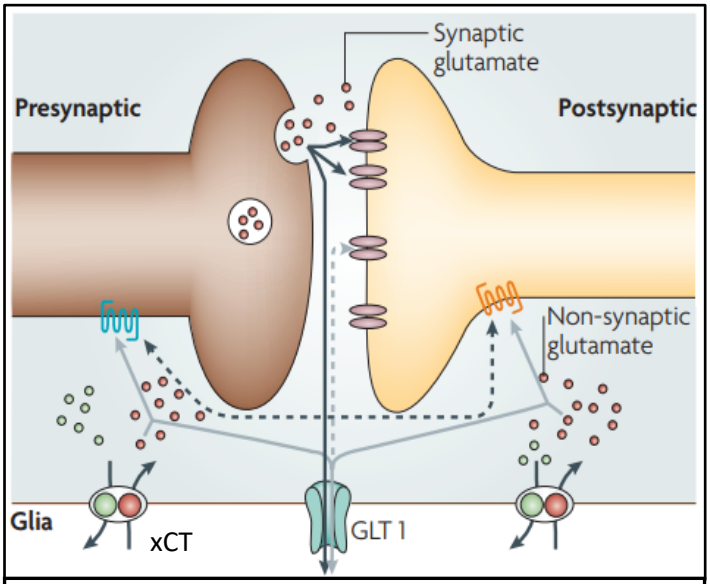

Figure 1.1. In drug-naïve conditions, glutamate homeostasis is maintained by xCT and GLT-1. Reprinted by permission from Springer Nature: Nature Reviews Neuroscience. The glutamate homeostasis hypothesis of addiction, Peter W. Kalivas (2009).

synaptic glutamate (Kalivas, 2009). Basal extracellular glutamate levels are regulated through the actions of the cystine-glutamate exchanger (Fig 1.1), which releases intracellular glutamate in a 1:1 stoichiometric exchange for extracellular cystine (McBean, 2002). The catalytic subunit of the cystine-glutamate exchanger (xCT) is predominantly expressed on astrocytes (Kalivas, 2009). In the NAc, astrocyte-derived cystine-glutamate exchange is hypothesized to account for ~60% of basal extracellular glutamate levels (Baker et al., 2002).

In addition to regulating extracellular glutamate levels, astrocytes play an important role in modulating synaptic glutamate levels, which is accomplished largely by glutamate uptake via the high affinity glutamate transporter GLT-1/EAAT2 (Fig 1.1). Likewise to system xC-, GLT-1 is also primarily expressed on astrocytes and accounts for approximately 90% of all glutamate clearance from the synapse (Danbolt, 2001).

In drug-naïve conditions, glutamate uptake within the synapse by GLT-1 prevents synaptically released glutamate from acting on extrasynaptically localized metabotropic

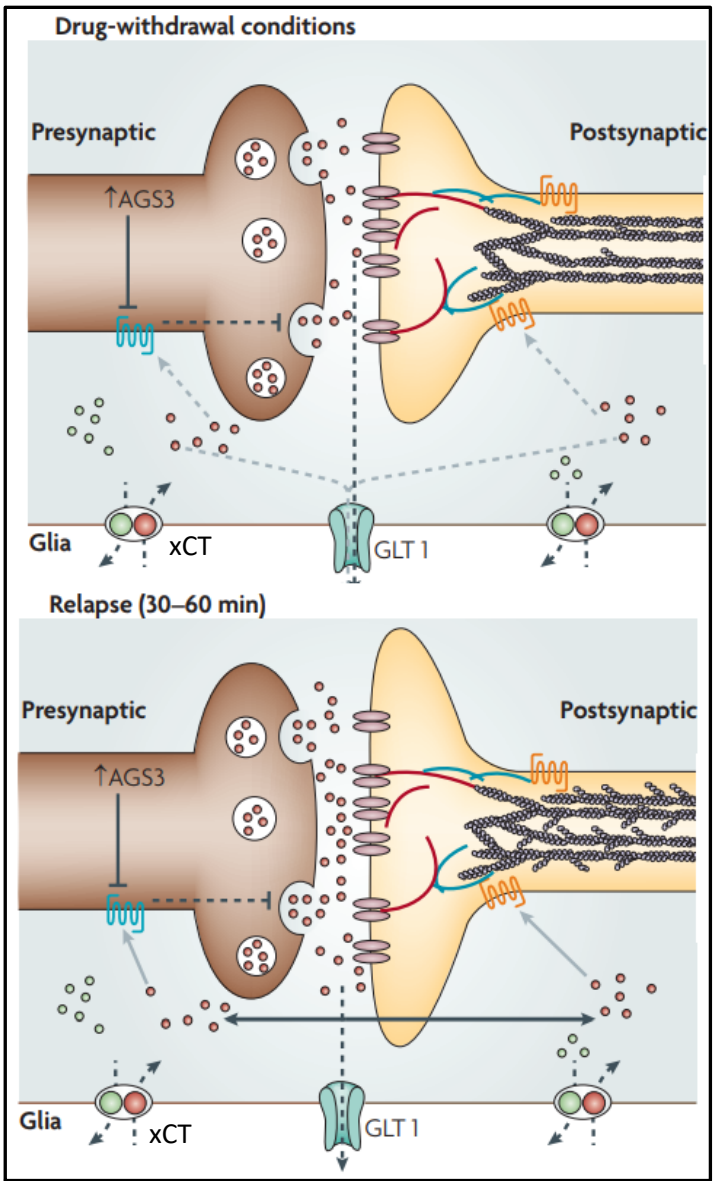

Figure 1.2. Withdrawal from cocaine self-administration leads to a downregulation in xCT and GLT-1 expression in the NAc (top), leading to enhanced synaptic potentiation during reinstatement (bottom). Reprinted by permission from Springer Nature: Nature Reviews Neuroscience. The glutamate homeostasis hypothesis of addiction, Peter W. Kalivas (2009).

glutamate receptors (mGluRs), and also limits extrasynaptically released glutamate from acting on synaptically located ionotropic glutamate receptors (iGluRs) (Kalivas, 2009). However, abstinence from cocaine self-administration has been demonstrated to disrupt glutamate homeostasis in the NAc by decreasing expression and activity of both xCT and GLT-1 (Fig 1.2 top) (Kalivas, 2009; Reissner and Kalivas, 2010; Scofield et al., 2016a; Scofield and Kalivas, 2014). For example, ShA cocaine self-administration and extinction training significantly decreases expression of xCT in the NAc (Knackstedt et al., 2010; Madayag et al., 2007). This decrease in cystine-glutamate exchange in the NAc leads to decreased basal levels of extracellular glutamate, and consequently reduced tone on inhibitory presynaptic mGluRs (mGluR 2/3), which in turn leads to heightened synaptic glutamate release, and

enhanced excitatory neurotransmission in the NAc (Fig 1.2 bottom) (Kalivas, 2009; Scofield and Kalivas, 2014).

Furthermore, numerous studies have also reported reductions in GLT-1 protein levels in the NAc following withdrawal from cocaine self-administration (Fig 1.2 top) (Fischer-Smith et al., 2012; Fischer et al., 2013; Knackstedt et al., 2010). Decreased GLT-1 expression leads to reduced uptake of synaptic glutamate, which in turn leads to enhanced glutamatergic transmission in the NAc (Scofield and Kalivas, 2014). Additionally, reduced expression of GLT-1 can lead to the increased ability of synaptically released glutamate to activate extrasynaptically located glutamate receptors, further enhancing synaptic potentiation in the NAc (Fig 1.2 bottom) (Kalivas, 2009; Scofield and Kalivas, 2014), and driving motor output.

Importantly, in the case of GLT-1, both the amount of cocaine self-administration and the duration of abstinence following cocaine self-administration correlate with decreases in GLT-1 protein levels in the NAc (Fischer-Smith et al., 2012). Longer access to cocaine self-administration (LgA, 6 hours/day vs. ShA, 2 hours/day) further reduces GLT-1 expression (Fischer-Smith et al., 2012). Furthermore, within both the ShA and LgA cocaine self-administration paradigms, longer abstinence periods (45 days vs. 1 day) augments the decrease in GLT-1 protein levels (Fischer-Smith et al., 2012). Therefore, rats that received the longest amount of cocaine self-administration (LgA) and underwent the longest duration of abstinence (45 days) showed the biggest decrease in NAc GLT-1 protein levels.

In agreement with these observations, preliminary data for this dissertation shows that LgA cocaine self-administration and prolonged abstinence leads to a decrease in GLT-1 mRNA levels in the NAc (Kim et al., 2018b). This decrease in GLT-1 mRNA was not seen following ShA cocaine self-administration and extinction training (Kim et al., 2018b), despite

known decreases in GLT-1 protein levels (Fischer-Smith et al., 2012; Fischer et al., 2013; Knackstedt et al., 2010). The combination of LgA cocaine self-administration and prolonged abstinence is akin to the incubation of cocaine craving model described above, and indicate that neuroadaptations that occur during the abstinence period following LgA cocaine self-administration may lead to increased drug seeking following a period of abstinence.

In summary, following withdrawal from cocaine self-administration, glutamate homeostasis is disrupted in the NAc primarily due to reductions in both xCT and GLT-1. In the case of GLT-1 protein expression, these decreases are potentiated by both duration of drug exposure and duration of abstinence. Furthermore, while GLT-1 protein expression is suppressed in each of these models, the LgA/abstinence model is the only self-administration model that leads to a decrease in GLT-1 mRNA levels and genetic regulation of GLT-1.

Astroglial modulators normalize glutamate homeostasis and reduce cocaine-seeking

As previously stated, xCT and GLT-1 are expressed on astrocytes, and downregulation of the two leads to disruptions in glutamate homeostasis and a subsequent increase in cocaine-seeking. Therefore, pharmacological compounds that restore expression of xCT and GLT-1, thereby restoring astrocyte functions such as glutamate uptake and cystine-glutamate exchange, represent potential pharmacotherapeutic treatment options for drug addiction. One such compound is the beta-lactam antibiotic ceftriaxone. Following ShA cocaine self-administration and extinction training, ceftriaxone restores expression of both xCT and GLT-1, and importantly, reduces both cue and cocaine-induced reinstatement (Knackstedt et al., 2010), a finding which has since been replicated by numerous studies (Bechard et al., 2018; Fischer et al., 2013; LaCrosse et al., 2017).

In addition to ceftriaxone, other drugs that enhance astrocyte functions also reduce cocaine-seeking. For example, N-acetylcysteine (NAC) is a cystine prodrug that enhances cystine-glutamate exchange (Lewerenz et al., 2013). NAC restores expression of both xCT and GLT-1 in the NAc following ShA cocaine self-administration and extinction training, and importantly, reduces reinstatement (Knackstedt et al., 2010; Reissner et al., 2015). The methylxanthine derivative propentofylline (PPF) is a glial modulator known to have neuroprotective effects by directly modulating the activity of glial cells (Sweitzer and De Leo, 2011). Similarly to ceftriaxone and NAC, PPF increases GLT-1 expression in the NAc and reduces cue-induced reinstatement (Reissner et al., 2014). Of significance, all three pharmacological compounds require restored expression of GLT-1 for the observed behavioral effects against reinstatement. If GLT-1 expression is suppressed in the NAc via antisense vivo-morpholino microinjections, the ability of these drugs to reduce cocaine-seeking is no longer observed, highlighting the significance of GLT-1 expression in the NAc in modulating drug-seeking behavior (LaCrosse et al., 2017; Reissner et al., 2014; Reissner et al., 2015). Accordingly, these data collectively contribute to a model in which decreased GLT-1 expression leads to an increase in cocaine-seeking, while restoration of GLT-1 expression lead to an attenuation of drug seeking.

Cocaine-induced changes in the morphology and synaptic colocalization of NAc astrocytes

In addition to its critical role in maintaining glutamate homeostasis, research over the past several years has demonstrated a vital role for astrocytes in numerous critical functions within the central nervous system (Allen and Barres, 2009; Chung et al., 2015; Khakh and Sofroniew, 2015; Papouin et al., 2017). In addition to providing structural and metabolic support to neurons, astrocytes play an important role in water and ion buffering, an active role

in the development and maturation of neuronal synapses, and transmitter uptake and release (Allen and Barres, 2009; Chung et al., 2015; Danbolt, 2001). Importantly, to this last point, astrocytes exhibit diverse branching processes which can extend toward and modulate synapses. Together, the synapse between a pre- and post-synaptic neuron and an astrocyte has been termed the "tri-partite" synapse (Araque et al., 1999; Heller and Rusakov, 2015).Within the tri-partite synapse, astrocytes can bidirectionally communicate with neurons and importantly, modulate synapses through the release of gliotransmitters such as glutamate, GABA and ATP (Bernardinelli et al., 2014; Blanco-Suarez et al., 2017). Dysfunction of astrocytes can consequently lead to the decreased ability of astrocytes to modulate synaptic functions. Likewise, alterations in markers for astrocyte structure and function have been implicated in a variety of neuropsychiatric and neurodegenerative diseases including depression (Dossi et al., 2018; Kim et al., 2018a), epilepsy (Dossi et al., 2018), Alzheimer's disease (Dossi et al., 2018; Garwood et al., 2017), Huntington's disease (Khakh et al., 2017) and Parkinson's disease (Booth et al., 2017).

The role of astrocytes in drug addiction remained largely unclear, since the changes in astrocytes in response to drug exposure have been shown to be dependent on both drug administration paradigm and brain region examined (Kim et al., 2018a). However, since xCT and GLT-1 are both expressed on astrocytes and expression is decreased in the NAc following withdrawal from cocaine self-administration, it is plausible to hypothesize that withdrawal from cocaine self-administration can result in parallel changes in the morphology of NAc astrocytes. Indeed, previous work from our laboratory has shown decreases in the surface area and volume of NAc astrocytes following ShA cocaine self-administration and extinction training (Scofield et al., 2016b; Testen et al., 2018). In addition to these changes in the

morphometric properties of astrocytes, the colocalization of NAc astrocytes with synaptic markers is also decreased (Scofield et al., 2016b; Testen et al., 2018). Moreover, these changes in astrocytes are specific to the NAc and requires the extinction training period following ShA cocaine self-administration (Testen et al., 2018).

The atrophy and decreased synaptic colocalization of NAc astrocytes may lead to the reduced ability of astrocytes to modulate NAc synapses, including the decreased ability of GLT-1 to uptake glutamate. This may then lead to an increase cocaine-seeking following a period of withdrawal. These results demonstrate a distinct change in the morphology and synaptic colocalization of NAc astrocytes following abstinence from ShA cocaine self-administration. However, it is unclear if these changes in NAc astrocytes are exacerbated following LgA cocaine self-administration and prolonged abstinence (similarly as GLT-1 expression). The overarching goal of Chapter 3 is to examine the changes in NAc astrocytes following LgA cocaine self-administration and prolonged abstinence.

While a well-defined correlation exists between GLT-1 expression and drug seeking, little is known about the relationship between GLT-1 expression and the cocaine-induced changes in NAc astrocytes. Previous work from our lab has shown that although ceftriaxone treatment did not ameliorate the cocaine-induced decrease in the surface area and volume of NAc astrocytes, it did however reverse the cocaine-induced decrease in synaptic colocalization of NAc astrocytes (Scofield et al., 2016b). These results suggest that increasing GLT-1 expression may drive peripheral astrocytic processes towards glutamatergic synapses, perhaps by normalizing extracellular glutamate levels, thereby reversing the cocaine-induced decrease in the synaptic colocalization of NAc astrocytes. As GLT-1 expression is primarily localized at the terminal points of peripheral astrocytic processes (Yang et al., 2009), this ceftriaxone-

induced reverse in synaptic colocalization may position GLT-1 directly at synapses, which will in turn lead to an increase in glutamate uptake and an attenuation in cocaine-seeking. Although these results are promising, ceftriaxone has a number of effects in addition to increased GLT-1 expression, including upregulation of xCT (Knackstedt et al., 2010). Therefore, it is not precisely clear how GLT-1 expression is involved. The goal of Chapter 4 is to directly and selectively examine the relationship between GLT-1 expression and the cocaine-induced changes in NAc astrocytes.

Sex differences in drug addiction

To date, the overwhelming majority of experiments examining the neurobiological mechanisms that underlie drug addiction have been in men and/or male animals. This is somewhat surprising considering the differences in males vs. females in drug intake. In humans, although the number of women diagnosed with SUD is lower than that of men, women transition to addiction faster than men and exhibit higher rates of relapse (Bobzean et al., 2014; Brady and Randall, 1999; Ignjatova and Raleva, 2009). Similar results have been reported in cocaine self-administration studies using female rats. For example, female rats generally exhibit higher lever pressing behavior during maintenance of cocaine self-administration, show greater escalation of cocaine-intake, and display increased cocaine-seeking behavior during a reinstatement test (Becker, 2016; Becker and Hu, 2008; Hu et al., 2004; Jackson et al., 2006; Lynch and Carroll, 1999; Swalve et al., 2016).

Moreover, numerous studies have shown that the estrous cycle in female rats can influence cocaine self-administration behavior. The rat estrous cycle, which lasts approximately four days, can be divided into four stages: metestrus, diestrus, proestrus and estrus (Cora et al., 2015; Marcondes et al., 2002). The proestrus stage is characterized by

elevated levels of hormones including: luteinizing hormone, follicle-stimulating hormone, progesterone, and estradiol. Roughly 12 hours following the start of proestrus, ovulation occurs during the estrus stage. Hormone levels then return to baseline levels during the metestrus and diestrus stages. Of the four stages, the estrus stage has been shown to most influence cocaine self-administration. Compared to the other stages of the estrous cycle, female rats in estrus exhibit a higher motivation to lever press for cocaine, and higher lever pressing behavior during a reinstatement test (Lacy et al., 2016).

Despite extensive studies conducted in male rats (Fischer-Smith et al., 2012; Fischer et al., 2013; Kim et al., 2018b; Knackstedt et al., 2010), the effects of cocaine on regulators of glutamate homeostasis have yet to be directly studied in female rats. One recent study has shown that the ability of ceftriaxone to attenuate cocaine-seeking in female rats is dependent on estrous cycle stage (Bechard et al., 2018). However, it is unknown if the estrous cycle has a direct effect on GLT-1 expression. Moreover, it is unclear how the estrous cycle interacts with the incubation of cocaine craving to modulate GLT-1 mRNA and protein levels in the NAc. The goal of Chapter 2 is to examine these questions and assess the changes in NAc GLT-1 mRNA and protein levels following LgA cocaine self-administration and prolonged abstinence in female rats.

Much like the literature on GLT-1 expression in female rats, there is a lack of investigation regarding the role of the estrous cycle in the structural and morphometric properties of astrocytes. Interestingly, one study has reported sex and estrous cycle-dependent changes in immunoreactivity of the astrocyte marker glial fibrillary acidic protein (GFAP) (Arias et al., 2009). This study found an increase in GFAP immunoreactivity in the hippocampus of female rats in estrus (Arias et al., 2009). It remains to be seen if similarly to

male rats (Scofield et al., 2016b; Testen et al., 2018), the morphology of NAc astrocytes changes in female rats following LgA cocaine self-administration and abstinence, and further, if the estrous cycle plays a role in the cocaine-induced changes in NAc astrocytes. One of the goals of Chapter 3 is to examine the changes astrocytes in the NAc of female rats following LgA cocaine self-administration and abstinence.

Specific Aims

As mentioned above, SUD is a chronic debilitating disorder affecting millions of people. However, no FDA approved treatment options exist for addiction to stimulants such as cocaine. To overcome this hurdle, a better understanding of the neurobiological changes that occur during abstinence from chronic drug use is needed. One promising candidate for a pharmacotherapeutic option is the upregulation of the astroglial glutamate transporter GLT-1. Although pharmacological drugs that upregulate GLT-1 expression have shown promise in animal studies, the results in human clinical trials have been mixed at best (Roberts-Wolfe and Kalivas, 2015). Therefore, to develop better pharmacotherapeutic treatments options for SUD, a better understanding of how cocaine regulates GLT-1 expression is needed. Moreover, although GLT-1 is primarily expressed on astrocytes, the relationship between GLT-1 and astrocytes is unclear. Therefore, the overarching goal of the experiments outlined in this dissertation is to examine in depth, the correlation between NAc GLT-1 expression and the cocaine-induced changes in the morphology and synaptic colocalization of NAc astrocytes.

Specific aim 1 will examine the changes in GLT-1 expression in the NAc of female rats following LgA cocaine self-administration and prolonged abstinence. Previous experiments have established that withdrawal from cocaine self-administration leads to a significant decrease in NAc GLT-1 protein in male rats (Fischer-Smith et al., 2012; Knackstedt et al., 2010). Furthermore, preliminary data for this dissertation indicates that following LgA cocaine self-administration and prolonged abstinence, GLT-1 mRNA levels are decreased in the NAc of male rats (Kim et al., 2018b). However, whether these above findings extend to female rats remains unknown. Furthermore, it is unclear on how the estrous cycle plays a role in the regulation of GLT-1 expression, and unknown if the estrous cycle is involved in the

cocaine-induced changes in GLT-1 expression in the NAc. Therefore, **specific aim 1a** will assess GLT-1 mRNA and **specific aim 1b** will examine GLT-1 protein levels in the NAc of female rats in different stages of the estrous cycle following LgA cocaine self-administration and prolonged abstinence. Specific aim 1 is described in Chapter 2.

Specific aim 2 will characterize the changes in the morphometric properties and synaptic colocalization of NAc astrocytes in both male and female rats following LgA cocaine self-administration and prolonged abstinence. A decrease in the surface area, volume and synaptic colocalization of NAc astrocytes has been well characterized following ShA cocaine self-administration and extinction training (Scofield et al., 2016b; Testen et al., 2018). However, LgA cocaine self-administration and prolonged abstinence leads to greater effects compared to the ShA/extinction model including: greater cocaine-seeking following abstinence (Li et al., 2015; Lu et al., 2004), synaptic changes in the NAc (Wolf, 2016), greater decreases in NAc GLT-1 protein (Fischer-Smith et al., 2012), and a decrease in NAc GLT-1 mRNA (Kim et al., 2018b). It is currently unknown if the changes observed in NAc astrocytes following ShA and extinction training persist, or are even augmented following LgA cocaine self-administration and prolonged abstinence. Therefore, **specific aim 2a** will assess the surface area, volume, and synaptic colocalization of NAc astrocytes in male rats, and **specific aim 2b** will examine these changes in the NAc of female rats following LgA cocaine self-administration and prolonged abstinence. Specific aim 2 is described in Chapter 3.

Specific aim 3 will examine the relationship between GLT-1 expression and the cocaine-induced changes in NAc astrocytes. Previous work from our laboratory has shown that ceftriaxone, a beta-lactam antibiotic that increases GLT-1 expression, can reverse the decrease in synaptic colocalization of NAc astrocytes following ShA cocaine self-

administration and extinction training (Scofield et al., 2016b). However, since ceftriaxone has a wide-range of effects, the direct contribution of restored GLT-1 expression to the ceftriaxone-induced reverse in synaptic colocalization remains unclear. **Specific aim 3a** will directly examine if similarly to pharmacological compounds that increase GLT-1, if viral overexpression of GLT-1 in the NAc using an adeno-associated virus (AAV) will also reduce cocaine-seeking following prolonged abstinence from LgA cocaine self-administration. **Specific aim 3b** will examine if AAV-induced overexpression of GLT-1 in the NAc can prevent the cocaine-induced changes in NAc astrocytes. Specific aim 3 is discussed in Chapter 4.

CHAPTER 2

GLT-1 EXPRESSION IN THE NAC OF FEMALE RATS FOLLOWING ABSTINENCE FROM LONG-ACCESS COCAINE SELF-ADMINISTRATION

Introduction

The nucleus accumbens (NAc) is a brain structure involved in the integration of limbic inputs and motor outputs, and thus plays a major role in many motivated behaviors related to drug addiction (Mogenson et al., 1980). As such, the cellular and molecular adaptations that occur in the NAc during abstinence from drug use represent candidate mechanisms that drive drug seeking and vulnerability to relapse (Hikida et al., 2016; Scofield et al., 2016a). Among these changes, alterations in glutamatergic signaling and glutamate homeostasis have been reported following exposure to drugs of abuse such as cocaine, heroin, nicotine, and alcohol (Kalivas, 2009; Scofield and Kalivas, 2014). Glutamate homeostasis is defined as the balance between synaptic and extra-synaptic glutamate levels, and is maintained by the cystine-glutamate exchanger and the glutamate transporter GLT-1, both of which are primarily expressed on astrocytes (Kalivas, 2009).

Cocaine self-administration and extinction training leads to decreased expression of the catalytic subunit of the cystine-glutamate exchanger (xCT), as well as GLT-1 expression in the NAc (Fischer-Smith et al., 2012; Knackstedt et al., 2010). Importantly, pharmacological compounds such as the beta-lactam antibiotic ceftriaxone, which restore expression of xCT and GLT-1, reduces cocaine-seeking (Knackstedt et al., 2010). In the case of GLT-1, longer access to cocaine self-administration (long access (LgA) vs. short access (ShA)), and longer

withdrawal periods (45 days vs. 1 day) leads to even further decreases in GLT-1 protein expression in the NAc (Fischer-Smith et al., 2012). Moreover, preliminary data for this dissertations shows that although both ShA and extinction training, as well as LgA and abstinence decreases GLT-1 protein expression in the NAc, only LgA cocaine self-administration and prolonged abstinence leads to a decrease in GLT-1 mRNA levels in the NAc (Kim et al., 2018b).

Although these changes in GLT-1 expression have been well characterized in the NAc following cocaine self-administration, all of these changes have been exclusively studied in male rats, with the exception of one study (Bechard et al., 2018). To date, it is currently unknown if these changes occur in the NAc of female rats, and further unknown if the estrous cycle plays a role in the cocaine-induced changes in GLT-1 expression. This is surprising considering the well-established behavioral differences in cocaine self-administration between male and female rats. For example, female rats show acquisition of cocaine self-administration at lower doses than male rats, exhibit an increase in lever-pressing during maintenance of cocaine self-administration, show higher motivation to lever-press for cocaine in a progressive ratio test, and display higher lever-pressing behavior during reinstatement tests (Bechard et al., 2018; Lacy et al., 2016; Lynch and Carroll, 1999; Swalve et al., 2016). Moreover, the estrous cycle potentiates cocaine self-administration behavior in female rats. Compared to other stages of the estrous cycle, female rats in estrus show higher breakpoint for cocaine in a progressive ratio test (Lacy et al., 2016), and also exhibited greater cocaine-seeking during reinstatement (Kippin et al., 2005).

In addition to these differences in cocaine self-administration behavior, numerous studies have reported sex differences in the brain's reward circuitry following exposure to

drugs of abuse. For example, female rats have higher concentrations of dopamine (DA) in the striatum following cocaine self-administration (Walker et al., 2006), an effect possibly mediated by sex differences in basal DA receptor levels (Becker and Hu, 2008). Moreover, the estrous cycle plays a role in the cocaine-induced sex differences in both dopaminergic activity and the enhanced behavioral response to cocaine. Using a cocaine conditioned place preference paradigm, one experiment demonstrated that female mice conditioned during the estrus stage showed an enhanced preference for cocaine (Calipari et al., 2017). Moreover, the same experiment showed that female mice in estrus exhibit enhanced basal DA firing in the ventral tegmental area and exhibit post-translational modifications at the DA transporter, both of which were hypothesized to lead to the enhanced preference for cocaine during the estrus stage (Calipari et al., 2017). Furthermore, using a cocaine self-administration paradigm, compared to cues that were presented when female mice were in diestrus, cocaine-related cues introduced during the estrus stage led to an enhanced motivation to lever press for cocaine (Johnson et al., 2019). Together, these results indicate that hormone levels during the estrus stage may drive neurobiological adaptations in female rats, leading to a heightened behavioral response to cocaine.

In addition to the changes in basal DA levels, numerous studies have also reported basal sex differences in glutamatergic signaling relevant to reward processing. Under baseline conditions in the hippocampus, female rats show a potentiation of AMPA receptor mediated currents, an effect hypothesized to be driven by phosphorylation of the GluA2 AMPA receptor subunit (Monfort et al., 2015). Furthermore, under basal conditions, female rats have increased expression of both NMDA and metabotropic glutamate receptors in the hippocampus, prefrontal cortex, and amygdala (Wang et al., 2015). In comparison to the literature describing

sex differences in neuroadaptations in DA signaling following exposure to cocaine, the literature on the sex differences in glutamatergic signaling after cocaine use is sparse. However, one study examined the effects of ceftriaxone in both male and female rats (Bechard et al., 2018). Similarly to previous studies in male rats (Fischer et al., 2013; Knackstedt et al., 2010), ceftriaxone treatment increased xCT and GLT-1 expression in the NAc of female rats following cocaine self-administration and extinction training. Moreover, also similarly to studies in male rats, ceftriaxone significantly attenuated cocaine-seeking in female rats (Bechard et al., 2018). However, when female rats were grouped according to estrous cycle stage, ceftriaxone had no effect on reinstatement behavior if female rats were tested during the estrus phase, an effect possibly driven by an increase in surface receptor expression of the glutamatergic AMPA receptor GluA1 (Bechard et al., 2018). These results indicate a possible correlation between hormone levels during the different stages of the estrous cycle and the cocaine-induced changes in glutamatergic signaling.

Given the lack of evidence regarding an association between abstinence from cocaine self-administration and changes in regulators of glutamate homeostasis, including GLT-1, in female rats, specific aim 1 of my dissertation was designed to test the hypothesis that LgA cocaine self-administration and abstinence would suppress GLT-1 mRNA and protein expression in females, similarly as in males. Since male rats show a significant decrease in both GLT-1 protein (Fischer-Smith et al., 2012; Knackstedt et al., 2010) and mRNA (Kim et al., 2018b) levels in the NAc following abstinence from LgA cocaine self-administration, I hypothesized that female rats will also show decrease in GLT-1 expression. Moreover, it is unknown if the fluctuations in hormone levels observed during the different stages of the estrous cycle influence NAc GLT-1 expression in the absence of cocaine. I hypothesized that

this decrease will be dependent on estrous cycle stage, and hormone levels will mediate the cocaine-induced decreases in NAc GLT-1 expression.

Methods

Animals

Female Sprague-Dawley rats (Envigo, Dublin, VA) aged approximately 6-8 weeks, and weighing between 175-200 g at the time of arrival were used in these experiments. All rats were individually housed under a reversed 12-hour light-dark cycle (7 a.m. off; 7 p.m. on) in temperature controlled clear plexiglass cages. All rats were allowed to acclimate to the animal facility for one week, where food and water were available ad libitum. Following acclimation, rats were placed on a food restricted diet of ~ 20 g of chow per day. Food restriction lasted throughout all surgical, post-operative, and food-training procedures. Rats were then returned to an ab libitum diet which lasted throughout the self-administration period and the duration of abstinence. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Jugular Vein Catheterization Surgery

Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). Prior to the start of surgical procedures, all rats were administered an i.p. injection of the analgesic meloxicam (4 mg/kg). Silastic catheters with a 22-gauge cannula (0.02 mm inner diameter, 0.047 mm outer diameter) were implanted into the rats' right jugular vein as previously described (Kim et al., 2018b; Sepulveda-Orengo et al., 2018). Rats were then given five days of post-operative care, where an antibiotic (gentamicin 5 mg/mL, 0.1 mL) and heparinized saline (30 units/kg, 0.1 mL) were administered i.v. through the implanted

catheter. Gentamicin and heparin were also administered throughout self-administration. Before the start of self-administration procedures, patency of the catheters was examined by administering a sub-threshold dose of propofol (10 mg/mL, 0.05 mL).

Behavioral Procedures

All food training and self-administration procedures were conducted in standard sound attenuated operant conditioning chambers (Med Associates, St. Albans VT). Prior to the start of self-administration, to facilitate acquisition to lever pressing, all rats were received one food training session, where lever presses on the active lever resulted in the delivery of one 45 mg food pellet (Bio Serv, Flemington, NJ). Food training sessions lasted a minimum of 6 hours and criteria for food training was set at greater than 100 responses on the active lever.

Following food training, rats were randomly assigned to two groups, where one group lever pressed for cocaine and the other group lever pressed for saline. As previously described (Kim et al., 2018b), all rats underwent LgA cocaine or saline self-administration for 6 hours/day on an FR1 schedule for 10 consecutive days. A response on the active lever resulted in the delivery of cocaine (0.75 mg/kg/infusion) or saline (0.9% sodium chloride), along with the presentation of audio (70 dB, 2.5 kHz tone) and visual (stimulus light above the active lever) cues for five seconds. A response on the active lever was followed by a 20-sec time out period. Active lever presses during this time resulted in no programmed responses. Responses on the inactive lever at any time during the session were recorded but also resulted in no programmed responses. Following 10 days of LgA cocaine or saline self-administration, all rats underwent experimenter-induced abstinence in the home cage for 45 days. Throughout the 45 days of abstinence, all rats were handled at least two times per week.

Cytological Assessment to Determine Estrous Cycle Phase

Following 45 days of abstinence from LgA cocaine or saline self-administration and prior to euthanasia, estrous cycle stage was determined for all rats using a previously described method (Marcondes et al., 2002). Briefly, a sterile swab moistened with saline was used to smear for phases of the estrous cycle. The smear was placed along a glass slide which was then stained using 1 mL of Wright's stain. The slices were then evaluated under 10x objective light microscope. Using previously defined criteria (Marcondes et al., 2002), the estrous cycle phase (metestrus, diestrus, estrus or proestrus) for each rat was determined. Similar to previous experiments (Bechard et al., 2018; Calipari et al., 2017), female rats in the metestrus and diestrus stages were both characterized as having low amounts of circulating hormone levels and thus, were combined into one group. The proestrus stage is characterized by elevated levels of circulating hormones, including increased levels of luteinizing hormone, follicle-stimulating hormone, progesterone and 17β- estradiol. The estrus stage is also characterized by increased levels hormones and is considered the stage at which ovulation occurs.

Experiment 2.1: Effects of LgA cocaine self-administration and prolonged abstinence on GLT-1 mRNA levels in the NAc of female rats

Tissue Collection and Sample Preparation

Twenty-four hours following the last day of abstinence, all rats were euthanized via rapid decapitation and tissue samples were collected from the NAc. All samples were stored in approximately 100 µL of RNA later solution (Qiagen, Germantown, MD) and stored at 4 °C until processing for qPCR.

qRT-PCR

100 µL of RNA from each NAc sample was isolated using the Trizol Plus RNA Purification Kit (ThermoFisher, Waltham, MA) according to instructions provided by the manufacturer. Five µL of RNA was used in a spectrophotometer to assess the purity and concentration of each RNA sample. Approximately 100-150 ng of RNA was then used in a reverse transcription assay and converted to cDNA. cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Waltham, MA). For each sample, qPCR amplification was performed in triplicate using the Taqman Fast Advanced Mastermix (Applied Biosciences, Waltham, MA) in a final volume of 20 µL (3 µL, ~15 ng of cDNA) under the following conditions: hold for 2 min at 50 °C, hold for 2 min at 95 °C, and 40 temperature cycles of 1 sec at 95 °C and 20 sec at 60 °C. For all samples, *GAPDH* was used as an endogenous control. Gene expression for the main isoform of GLT-1 (GLT-1A), as well as a splice isoform of GLT-1 (GLT-1B) were examined. All sequences for GLT-1 and GAPDH primers/probes were identical to previous sequences used to assess GLT-1 mRNA levels in the NAc of male rats (Kim et al., 2018b). The sequences were as follows: GLT-1A Forward: GGAAAGCAACTCTAATCAGTG, Reverse: CATTGGCCGCCAGAGTTAC, Probe: FTCAATGCCGCACACAACTCTGTCGQ; GLT-1B Forward: GGAAAGCAACTCTAATCAGTG, Reverse: GGAAAGCAACTCTAATCAGTG, Reverse: TCCAGGAATGGGAAAGGTAC, Probe: FTCAATGCCGCACACAACTCTGTCGQ; GAPDH Forward: AGGTCGGTGTGAACGGATTT, Reverse: GGCAACAATGTCCACTTTGT, Probe: FCGCCTGGTTACCAGGGCTGCCQ (F = 5' Fluorescein (FAM); Q = Quencher (TAMRA)).

Data Analysis

All statistical analysis was conducted using SigmaPlot 11.0 or SPSS (version 25) software. For all self-administration behavioral data, a mixed ANOVA was run with drug (saline vs. cocaine) and time (self-administration session) set as factors. For all behavioral measures, the dependent variable was the amount of cocaine or saline infusions received, or the amount of active lever presses. For GLT-1 gene expression analysis, the ΔΔCt method was used as previously described (Kim et al., 2002). Relative concentrations of GLT-1A and GLT-1B were examined using GAPDH as an endogenous control in saline vs. cocaine self-administering rats. To determine overall changes in GLT-1 mRNA levels in saline vs. cocaine rats (regardless of estrous cycle stage), a two-tailed, unpaired *t*-test was run to examine differences between saline vs. cocaine groups in GLT-1A and GLT-1B gene expression. To assess the role of the estrous cycle in the cocaine-induced changes in GLT-1 mRNA levels, a two-way ANOVA was employed with drug (saline vs. cocaine) and estrous cycle stage (metestrus/diestrus vs. estrus vs. proestrus) set as factors.

Experiment 2.2: Effects of LgA cocaine self-administration and prolonged abstinence on GLT-1 protein levels in the NAc of female rats

Tissue Collection and Sample Preparation

Twenty-four hours following the last day of abstinence, all rats were euthanized via rapid decapitation and 1.5 mm punches surrounding NAc tissue collected. NAc tissue was then 8 stroked in a hand-driven glass-teflon homogenizer. NAc tissue was homogenized in 400 µL of sucrose buffer containing 1:100 protease/phosphatase inhibitors (Thermo Fisher, Waltham, MA) and 4 µl 0.5 M EDTA (Thermo Fisher, Waltham, MA). A P2 membrane subfraction was prepared as previously described (Knackstedt et al., 2010). Briefly, the homogenate mixture

was centrifuged at 1,000 x g for 10 min. The supernatant was removed, transferred to a microcentrifuge tube and centrifuged at 12,000 x g for 20 min. The cytosolic fraction was removed and the remaining P2 pellet was stored at -80 °C until use.

Western blot

For Western blot, P2 pellets were thawed on ice, and resuspended in 35 µL of RIPA buffer containing 1% sodium dodecyl sulfate (SDS), protease/phosphatase inhibitors, and EDTA. Samples were then centrifuged at 14,000 x g for 10 min. Protein concentration for each sample was then determined using a BCA assay (Pierce Microplate BCA Protein Assay Kit, ThermoFisher, Waltham, MA). Equal amounts of protein were prepared of 4x NuPage sample buffer (Thermo Fisher, Waltham, MA), along with 20 µL of β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and heated at 50 °C for 30 min prior to loading on a 10% criterion Tri-HCl gel (Bio-Rad, Hercules, CA). Samples were then transferred onto PVDF membranes (Millipore Sigma, Burlington, MA). Membranes were blocked for 1 hour at room temperature in Licor Odyssey blocking buffer, and incubated with primary antibodies overnight at 4 °C (GLT-1: Millipore AB1783 at 1:4000; Calnexin: Enzo ADI-SPA-860 at 1:4000). Secondary antibody incubation was performed for 1.5 hours at 4 °C (Licor 800 CW anti-guinea pig at 1:15,000; Licor 680 RP anti-rabbit at 1:15,000). Membranes were then washed 3 x 5 min in TBS + 0.1% tween and imaged on a Licor Odyssey Fc imager. Relative GLT-1 levels were determined using a ratio of GLT-1 to calnexin. Values were normalized to saline self-administering rats in order to determine relative changes in GLT-1 protein expression in saline vs. cocaine self-administering rats.

Data Analysis

All statistical analysis was conducted using SigmaPlot 11.0 or SPSS (version 25) software. For all self-administration behavioral data, a mixed ANOVA was run with drug (saline vs. cocaine) and time (self-administration day) set as factors. For all behavioral measures, the dependent variable was the amount of cocaine or saline infusions received, and the amount of active lever presses. To determine overall changes in GLT-1 protein levels in saline vs. cocaine rats (regardless of estrous cycle stage), a two-tailed, unpaired *t*-test was used. To assess if the estrous cycle is involved in the cocaine-induced changes in GLT-1 protein, a two-way ANOVA was used with drug (saline vs. cocaine) and estrous cycle stage (metestrus/diestrus vs. estrus vs. proestrus) set as factors.

Results

Experiment 2.1: Effect of LgA cocaine self-administration and prolonged abstinence on GLT-1 mRNA levels in the NAc of female rats

Figure 2.1 shows self-administration data for all rats across the ten days of self-administration. For active lever presses (Fig 2.1a), there was a significant main effect of drug ($F_{(1, 389)} = 52.42$, $p < 0.001$) and a significant main effect of self-administration session ($F_{(9, 389)} = 2.13$, $p < 0.05$). The interaction between drug and session was not statistically significant ($F_{(9, 389)} = 0.92$, $p = 0.51$). Post-hoc comparisons using the Holm-Sidak method reveal that on all self-administration sessions, cocaine self-administering rats exhibited a significantly greater amount of active lever responses vs. saline self-administering rats ($p < 0.05$).

For infusions received during self-administration sessions (Fig 2.1b), the main effect of drug ($F_{(1, 389)} = 172.56$, $p < 0.001$) and the main effect of time (self-administration session)

were significant ($F_{(9, 389)} = 5.92$, $p < 0.001$). The interaction between drug and self-administration session was also statistically significant ($F_{(9, 389)} = 43.59$, $p < 0.001$). Post-hoc comparisons using the Holm-Sidak method reveal that on all self-administration sessions, cocaine self-administering rats exhibited a significantly greater amount of infusions received vs. saline self-administering rats ($p < 0.05$). Furthermore, within cocaine self-administering rats, post-hoc comparisons reveal a significant increase in the number of infusions received on day 9 (vs. day 1: $t = 12.95$, $p < 0.001$; vs. day 2: $t = 11.13$, $p < 0.001$) on day 10 (vs. day 1: $t = 12.95$: $p < 0.001$; vs. day 2: $t = 10.84$, $p < 0.001$). This escalation of intake was not observed in saline self-administering rats.

To examine overall changes in GLT-1A and GLT-1B mRNA levels in saline vs. cocaine female rats (regardless of estrous cycle stage), a two-tailed, unpaired *t* test was used. There were no statistically significant differences between saline and cocaine self-administering female rats in NAc expression of the main isoform of GLT-1, GLT-1A ($t_{(37)} = 0.42$, $p = 0.68$, Fig 2.2a). Furthermore, there was also no difference in gene expression of the splice isoform of GLT-1, GLT-1B ($t_{(36)} = 0.04$, $p = 0.96$, Fig 2.2b) levels.

To assess a possible interaction between the estrous cycle and cocaine-induced changes in GLT-1A or GLT-1B mRNA levels, a two-way ANOVA was employed with drug (saline vs. cocaine) and estrous cycle stage (met/diestrus vs. estrus vs. proestrus) set as factors. For GLT-1A gene expression, there was no significant main effect of drug ($F_{(1, 38)} = 1.52$, $p = 0.23$) or estrous cycle stage ($F_{(2, 38)} = 0.11$, $p = 0.89$). The interaction between drug and cycle was also not significant ($F_{(1, 38)} = 0.07$, $p = 0.93$). For GLT-1B mRNA levels, there was no significant main effect of drug ($F_{(1, 37)} = 4.09$, $p = 0.052$) or estrous cycle stage ($F_{(1, 38)} = 2.94$, $p = 0.067$). The interaction between drug and cycle was also not significant ($F_{(2, 37)} = 3.00$, p

= 0.064). These results indicate that female rats show no change in GLT-1 mRNA levels in the NAc following LgA cocaine self-administration and abstinence. Moreover, the lack of any changes in GLT-1 gene expression at different stages of the estrous cycle in saline self-administering rats suggests that even in the absence of cocaine, hormone levels during the estrous cycle have no effect on GLT-1 mRNA levels.

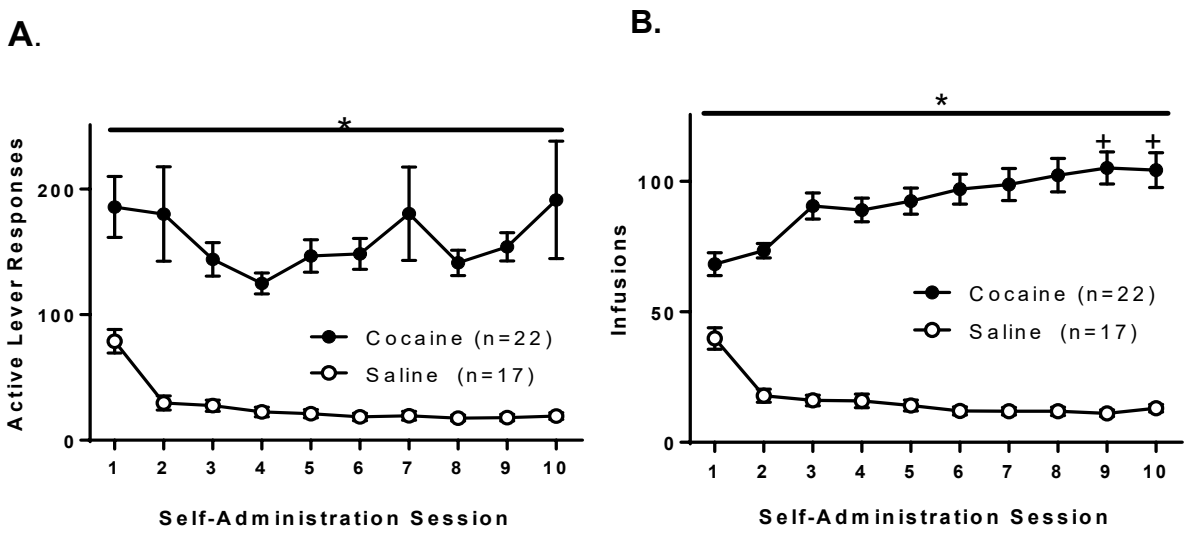

Figure 2.1 Behavioral data for all female rats used to assess NAc GLT-1 mRNA levels following LgA self-administration and prolonged abstinence. (A) active lever presses and **(B)** infusions received during self-administration sessions in saline vs. cocaine self-administering female rats. Female rats lever pressing for cocaine show a significant increase in the number of active lever presses and infusions received throughout all self-administration sessions (* cocaine vs. saline rats, $p < 0.05$). Female rats lever pressing for cocaine also exhibit an escalation of cocaine intake (+ days 9 and 10 vs. days 1 and 2, $p < 0.05$).

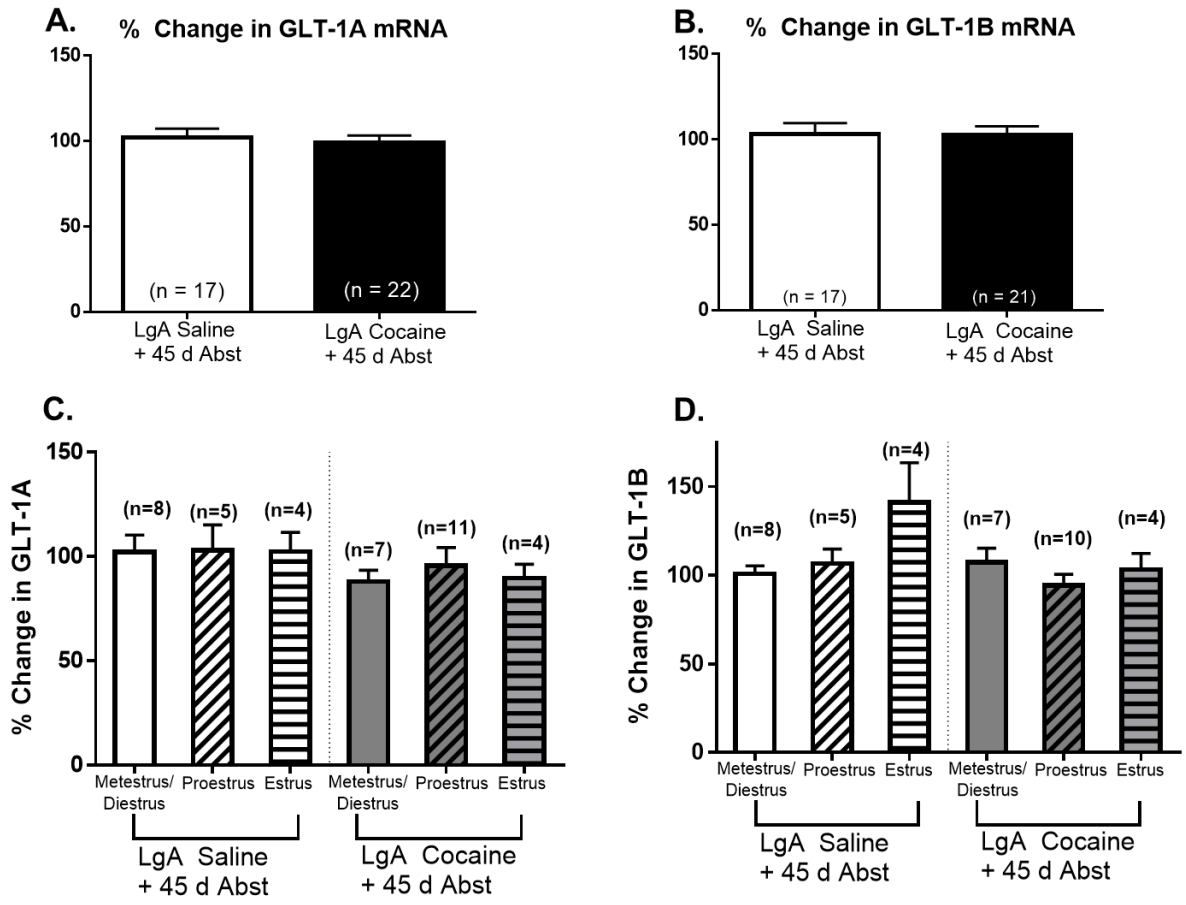

Figure 2.2 NAc GLT-1 gene expression in female rats after LgA cocaine self-administration and prolonged abstinence. (A) mRNA levels of the main isoform of GLT-1, GLT-1A in saline vs. cocaine self-administering rats **(B)** mRNA levels of GLT-1B, a splice isoform of GLT-1, in saline vs. cocaine self-administering rats. **(C)** GLT-1A mRNA levels in saline vs. cocaine self-administering rats divided into groups based on estrous cycle stage. **(D)** GLT-1B mRNA levels in saline vs. cocaine self-administering rats divided into groups based on estrous cycle stage. No significant changes were observed in NAc GLT-1 mRNA levels in female rats following LgA cocaine self-administration and prolonged abstinence.

Experiment 2.2: Effect of LgA cocaine self-administration and prolonged abstinence on GLT-1 protein levels in the NAc of female rats

Figure 2.3 shows self-administration data for all female rats used to assess GLT-1 protein levels in the NAc following LgA cocaine self-administration and prolonged abstinence. For active lever presses (Fig 2.3a), there was a significant main effect of drug ($F_{(1, 309)} = 43.34$, $p < 0.001$) but not self-administration session ($F_{(9, 309)} = 1.44$, $p = 0.17$). The interaction between drug and session was also not statistically significant ($F_{(9, 309)} = 0.81$, $p = 0.59$). Post-hoc comparisons using the Holm-Sidak method reveal that on all self-administration sessions, cocaine self-administering rats exhibited a significantly greater amount of active lever responses vs. saline self-administering rats ($p < 0.05$).

For infusions received during self-administration sessions (Fig 2.3b), there was a significant main effect of drug ($F_{(1, 309)} = 616.08$, $p < 0.001$) and a significant main effect of self-administration session ($F_{(9, 309)} = 12.88$, $p < 0.001$). The interaction between drug and time was also statistically significant ($F_{(9, 309)} = 56.55$, $p < 0.001$). Post-hoc comparisons using the Holm-Sidak method reveal that on all self-administration sessions, cocaine self-administering rats exhibited a significantly greater amount of infusions received vs. saline self-administering rats ($p < 0.05$). Furthermore, within cocaine self-administering rats, post-hoc comparisons reveal a significant increase in the number of infusions received on day 9 (vs. day 1: $t = 13.28$, $p < 0.001$; vs. day 2: $t = 12.44$, $p < 0.001$) on day 10 (vs. day 1: $t = 15.6$: $p < 0.001$; vs. day 2: $t = 14.75$, $p < 0.001$). This escalation of intake was not observed in saline self-administering rats.

To examine overall changes in GLT-1 protein expression in saline vs. cocaine self-administering female rats (regardless of estrous cycle stage), a two-tailed, unpaired *t* test was

used. There was no statistically significant difference between saline and cocaine self-administering female rats in NAc GLT-1 expression ($t_{(29)} = -1.62$, $p = 0.12$, Fig 2.4a and Fig 2.4b). To assess an interaction between the estrous cycle and the cocaine-induced changes in GLT-1 protein expression, a two-way ANOVA was used with drug (saline vs. cocaine) and estrous cycle stage (met/diestrus, estrus, proestrus) set as factors. There was no significant main effect of drug ($F_{(1, 30)} = 1.57$, $p = 0.22$) or cycle ($F_{(2, 30)} = 0.24$, $p = 0.79$). The interaction between drug and cycle was also not statistically significant ($F_{(1, 30)} = 0.19$, $p = 0.82$). These results indicate that similarly to NAc GLT-1 mRNA levels, female rats show no change in GLT-1 protein expression in the NAc following LgA cocaine self-administration and prolonged abstinence. Furthermore, identically to GLT-1 gene expression, these results also show that in the absence of cocaine, hormone levels during the estrous cycle in saline self-administering female rats have no effect on GLT-1 protein expression in the NAc.

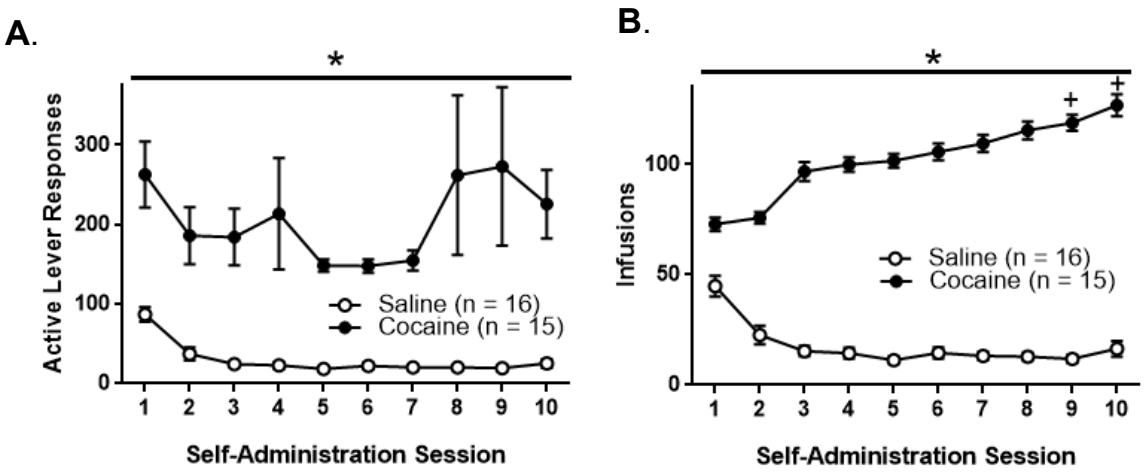

Figure 2.3 Behavioral data for all female rats used to assess NAc GLT-1 protein levels following LgA self-administration and prolonged abstinence (A) active lever presses and **(B)** infusions received during self-administration sessions in saline vs. cocaine self-administering rats. Rats lever pressing for cocaine show a significant increase in the number of active lever presses and infusions received throughout all self-administration sessions (* cocaine vs. saline rats, $p < 0.05$). Rats lever pressing for cocaine also exhibit an escalation of cocaine intake (+ days 9 and 10 vs. days 1 and 2, $p < 0.05$).

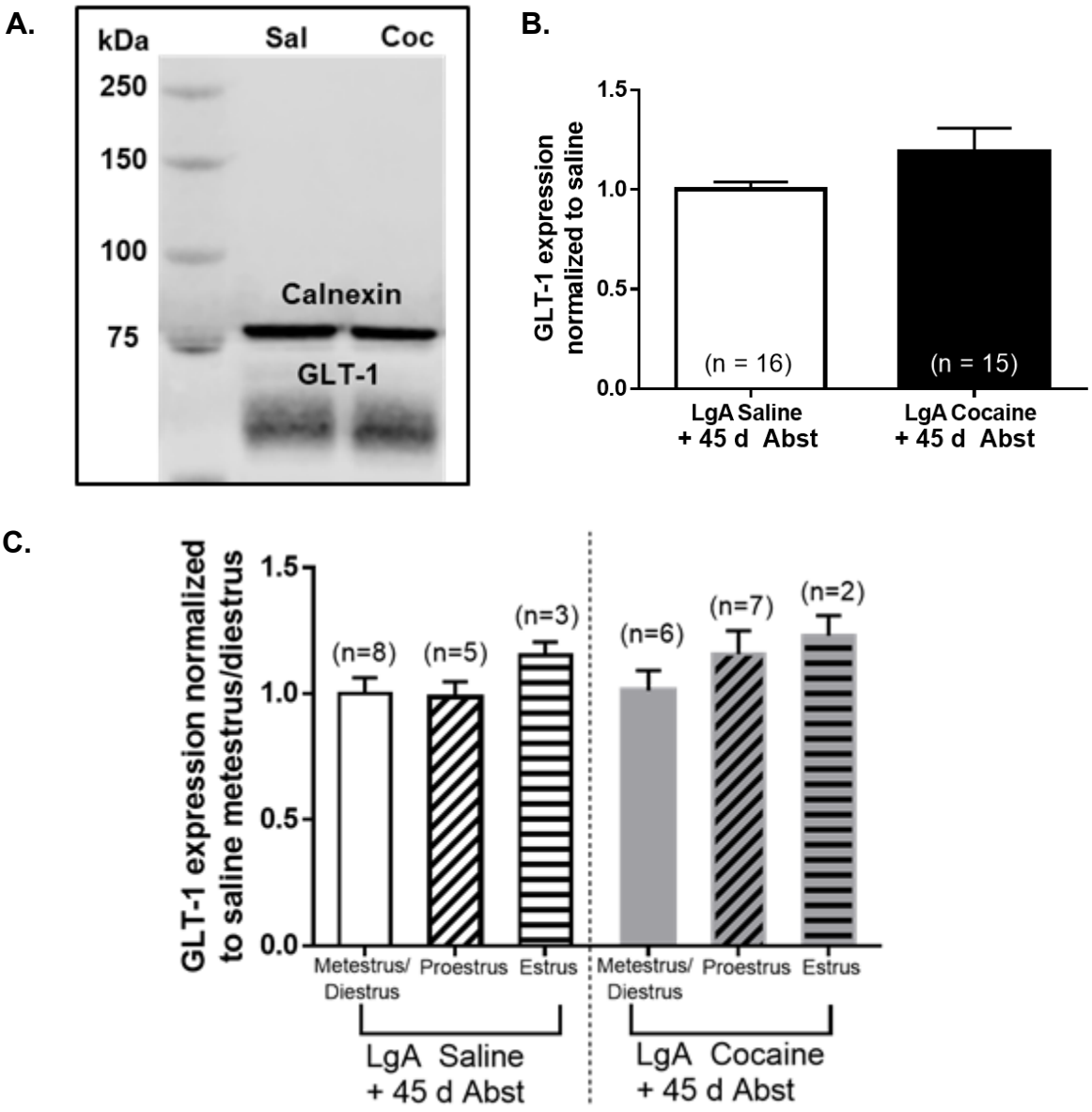

Figure 2.4 NAc GLT-1 protein expression in female rats after LgA cocaine self-administration and prolonged abstinence (A) Representative Western blot for calnexin (top band) and GLT-1 (bottom band) in rats that self-administered saline (Sal) or cocaine (Coc) **(B)** Relative NAc GLT-1 protein levels in saline vs. cocaine self-administering rats **(C)** Relative NAc GLT-1 protein levels in saline vs. cocaine self-administering rats were divided into groups based on estrous cycle stage. No statistically significant differences were observed in NAc GLT-1 protein expression in female rats.

Discussion

The results from this chapter indicate that GLT-1 mRNA and protein levels are unchanged in the NAc of female rats following LgA cocaine self-administration and prolonged abstinence. This result is in contrast to a previous experiment in male rats which showed a pronounced downregulation in GLT-1 protein expression (Fischer-Smith et al., 2012). These results are also in contrast to preliminary data for this dissertation which showed a decrease in GLT-1 mRNA levels in male rats (Kim et al., 2018b). Despite evidence that GLT-1 protein expression is decreased in the NAc of female rats following ShA cocaine self-administration and extinction training (Bechard et al., 2018), the results from this chapter indicate that downregulation of GLT-1 is not a neurobiological adaptation that occurs in the NAc of female rats during abstinence from LgA cocaine self-administration. Therefore, in female rats, other mechanisms may contribute to the incubation of cocaine craving.

The absence of any changes in NAc GLT-1 expression in female rats may indicate that sex hormones may be protective against cocaine-induced downregulation of NAc GLT-1 expression. However, the results of experiments 2.1 and 2.2 show that there was no effect of estrous cycle stage and moreover, no interaction between estrous cycle stage and cocaine on GLT-1 mRNA or protein levels. Nevertheless, in these two experiments, the estrous cycle was only monitored and not manipulated. This approach was utilized over traditional experiments investigating sex differences in drug addiction, which have typically utilized ovariectomized rats (Becker, 2016). In these cases, ovariectomized rats are typically administered a bolus dose of sex hormones. Although these studies have contributed greatly to the fields understanding of the neurobiological mechanisms underlying sex differences in drug addiction, bolus administration of hormones does not model the natural fluctuations in hormone levels observed

throughout the different stages of the estrous cycle. Furthermore, in experiments 2.1 and 2.2, estrous cycle stage was only recorded directly before euthanasia. Therefore, the possibility remains that fluctuating hormone levels throughout the different stages of the estrous cycle may have an influence on NAc GLT-1 expression in female rats, but this effect was unable to be observed in experiments 2.1 and 2.2. In support of this hypothesis, a previous study has shown that although ceftriaxone reduces cocaine-seeking in male rats and non-estrus female rats, it was unable to reduce reinstatement in female rats that were tested during the estrus stage (Bechard et al., 2018). Compared to the metestrus and diestrus stages, the estrus stage is characterized by high circulating hormone levels, including increased levels of 17β-estradiol (Marcondes et al., 2002). Since ceftriaxone is known to attenuate cocaine-seeking in part by restoring GLT-1 expression (Fischer et al., 2013; LaCrosse et al., 2017), it remains possible that elevated hormone levels during the estrus stage may protect against the downregulation of NAc GLT-1 levels. In this case, female rats in estrus may show augmented GLT-1 levels, thereby reducing the efficacy of ceftriaxone in increasing GLT-1 and subsequently attenuating cocaine-seeking.

In support of the hypothesis that elevated sex hormone levels can protect against the loss of GLT-1, numerous *in vitro* studies suggest that estrogens can upregulate GLT-1 expression (Liang et al., 2002; Pawlak et al., 2005). Indeed, numerous studies have reported that estrogens such as estradiol and tamoxifen can act as neuroprotective agents by increasing expression of glutamate transporters, thereby normalizing glutamate uptake (Kimelberg et al., 2000; Lee et al., 2009; Liang et al., 2002; Pawlak et al., 2005; Shi et al., 1997). For example, administration of 17β-estradiol in cultured astrocytes significantly increased expression of both GLT-1 mRNA and protein, and further, increased uptake of l-glutamate (Pawlak et al., 2005).

Similarly, 17β-estradiol increased GLT-1 expression and increased glutamate uptake in astrocytes cultured from patients with Alzheimer's disease (Liang et al., 2002). Furthermore, both 17β-estradiol and tamoxifen were shown to be protective against manganese-induced neurotoxicity and loss of glutamate uptake (Lee et al., 2009). This was accomplished by increasing expression and activity of glutamate transporters via activation of the MAPK, extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways (Lee et al., 2009). These studies all indicate that estrogens such as 17β-estradiol and tamoxifen can increase expression of glutamate transporters, thereby increasing glutamate uptake and protecting against glutamate-induced excitotoxicity. However, since these studies were all conducted *in vitro*, it remains to be seen if the neuroprotective properties of estrogens are observed *in vivo*. Despite significant decreases in NAc GLT-1 mRNA and protein levels in male rats (Fischer-Smith et al., 2012; Kim et al., 2018b), the absence of any changes in NAc GLT-1 expression in female rats suggests that estrogens may protect against the loss of GLT-1 following LgA cocaine self-administration and abstinence. However, additional experiments are needed to confirm this hypothesis.

In addition to the possibility that elevated hormone levels may protect against the downregulation of GLT-1 expression, basal differences in glutamatergic transmission between male and female rats may underlie the sex difference in GLT-1 expression following LgA cocaine self-administration and abstinence. Compared to male rats, female rats show increased basal levels of AMPA and NMDA receptors, as well as higher levels of the metabotropic glutamate receptors mGluR2/3 and mGluR5 (Monfort et al., 2015; Wang et al., 2015). Basal sex differences in the expression of glutamate receptors may lead to altered glutamatergic signaling, which in turn can lead to differential expression of glutamate transporters including

GLT-1 in male vs. female rats. In male rats, withdrawal from cocaine self-administration has been shown to lead to decreased basal levels of extrasynaptic glutamate (McFarland et al., 2003). This decrease in basal glutamate levels leads to reduced tone on presynaptic mGluR2/3, which in turn, increases glutamate release when animals are re-exposed to cocaine-related cues (Kalivas, 2009). In comparison to male rats, the higher basal levels of mGluR2/3 observed in female rats may lead to attenuated glutamate release and altered glutamatergic signaling in the NAc of female rats during abstinence from cocaine self-administration. Nonetheless, further experiments are needed to directly examine the contribution of basal sex differences in glutamatergic signaling in GLT-1 expression following cocaine self-administration.

As previously mentioned, GLT-1 is primarily expressed on astrocytes (Danbolt, 2001). Any changes in GLT-1 expression may then indicate impaired physiology of NAc astrocytes or vice versa. In addition, astrocytes express the α and β forms of the estrogen receptor, both of which have been shown to play a vital role in the neuroprotective properties of estrogen (Azcoitia et al., 2001; Dhandapani and Brann, 2003; Dhandapani and Brann, 2007). Given the difference between male and female rats in GLT-1 expression following LgA cocaine self-administration and abstinence, as well as the role of astrocytes in the neuroprotective properties of estrogen, it then becomes imperative to examine the structural and morphometric properties of astrocytes in male vs. female rats. These experiments are discussed in Chapter 3.

In summary, the results from this chapter show that following LgA cocaine self-administration and prolonged abstinence, GLT-1 mRNA (experiment 2.1) and protein expression (experiment 2.2) are not changed in the NAc of female rats. This is despite ample evidence that indicates a significant downregulation of both GLT-1 mRNA and protein levels in the NAc of male rats following LgA cocaine self-administration and abstinence.

Furthermore, pharmacological restoration of GLT-1 is therapeutic in many disease states including: drug addiction (Fischer et al., 2013; Knackstedt et al., 2010), epilepsy (Sha et al., 2017; Soni et al., 2015), amyotrophic lateral sclerosis (Banasr et al., 2010; Brothers et al., 2013), Parkinson's disease (Pajarillo et al., 2019) and Alzheimer's disease (Soni et al., 2014). However, the lack of any significant changes in GLT-1 expression in female rats suggests that hormone levels during the estrous cycle may protect against loss of NAc GLT-1. However, since the estrous cycle was only monitored in experiments 2.1 and 2.2, future studies can manipulate hormone levels during self-administration and abstinence to directly examine this relationship. Furthermore, since GLT-1 and estrogen receptors are expressed on astrocytes, it becomes critical to examine the structural properties of astrocytes, as well as the relationship between astrocytes and neuronal synapses following LgA cocaine self-administration and abstinence in both male and female rats. These experiments are described in detail below in Chapter 3.

CHAPTER 3

SEX DIFFEENCES IN THE MORPHOMETRIC PROPERTIES AND SYNAPTIC COLOCALIZATION OF NAC ASTROCYTES FOLLOWING ABSTINENCE FROM LGA COCAINE SELF-ADMINISTRATION

Introduction

As discussed in Chapter 2, the neurobiological adaptations that occur in the NAc during withdrawal from drug use represent potential mechanisms that drive drug seeking and vulnerability to relapse. Among these changes, impairments in glutamate homeostasis occur in the NAc after cocaine self-administration (Fischer-Smith et al., 2012; Kalivas, 2009; Kim et al., 2018b; Knackstedt et al., 2010). In particular, expression of the astroglial glutamate transporter GLT-1, as well as expression of xCT, the catalytic subunit of the cystine glutamate exchanger, are decreased following ShA cocaine self-administration and extinction training (Knackstedt et al., 2010). Importantly, pharmacological restoration of these proteins impairs reinstatement of cocaine seeking (Knackstedt et al., 2010; Reissner et al., 2014; Reissner et al., 2015; Sepulveda-Orengo et al., 2018). Notably, both GLT-1 and xCT are primarily expressed on astrocytes (Danbolt, 2001; Ottestad-Hansen et al., 2018; Scofield and Kalivas, 2014). The downregulation of astrocytic proteins such as GLT-1 and xCT in the NAc following ShA cocaine self-administration and extinction training raises the question of what other adaptations may also occur in astrocytes during withdrawal from cocaine self-administration.

Previous studies that have examined cocaine-induced changes in astrocytes have typically relied on immunoassays for glial fibrillary acidic protein (GFAP) (Bowers and

Kalivas, 2003; Fattore et al., 2002; George et al., 2008). GFAP is an intermediate filament protein and is a cytoskeletal marker for astrocytes (Hol and Pekny, 2015; Middeldorp and Hol, 2011). The effects of cocaine on GFAP expression are dependent on administration paradigm and brain region examined (Kim et al., 2018a). For example, numerous studies have reported increases in GFAP expression after non-contingent cocaine administration in brain regions including the hippocampus (Blanco-Calvo et al., 2014), striatum (Periyasamy et al., 2016) and NAc (Bowers and Kalivas, 2003). In contrast, other studies have reported no change in GFAP expression in the same brain regions (Bowers and Kalivas, 2003; Cappon et al., 1998; El Hage et al., 2012; Fattore et al., 2002). A more recent study reported decreased GFAP expression following contingent cocaine exposure, finding reduced GFAP protein expression in the NAc following 14 – 16 days of extinction training from ShA cocaine self-administration (Scofield et al., 2016b). In addition to quantifying changes in GFAP expression, GFAP immunostaining has also been used to examine changes in the morphology of astrocytes after cocaine exposure (Fattore et al., 2002). For example, after 7 and 14 days of 20 mg/kg i.p. injections of cocaine, Fattore et al (Fattore et al., 2002), reported a significant reduction in the area and length of astrocytic processes in the hippocampus.

Although the studies above have contributed to the understanding of how astrocytes change after cocaine exposure, immunoassays for GFAP are not without limitations. For example, approximately 50% of astrocytes do not express GFAP (Kettenmann and Verkhratsky, 2011; Kimelberg, 2004). Importantly, this number has also been shown to vary dependent on brain region (Sofroniew, 2009; Sofroniew and Vinters, 2010). Moreover, GFAP only constitutes about 15% of the total volume of an astrocyte (Benediktsson et al., 2005;

Rajkowska and Stockmeier, 2013), making it difficult to assess changes in astrocyte morphology with great detail.

To this end, our lab has employed a novel approach to examine the morphometric properties and synaptic colocalization of astrocytes in their entirety (Scofield et al., 2016b; Testen et al., 2019; Testen et al., 2018). This approach utilizes a membrane-tagged lymphocyte protein tyrosine kinase-green fluorescent protein (Lck-GFP) virus under the control of an astrocyte specific GfaABC1D promoter. Post-translational modifications on the N-terminus of Lck tether the protein to the plasma membrane of the cell (Shigetomi et al., 2013; Shigetomi et al., 2010). When under control of the GfaABC1D promoter, this allows for visualization of the entire astrocyte, including its fine peripheral processes, which are unable to be observed using traditional GFAP immunostaining. Combined with high resolution confocal microscopy, single, isolated Lck-GFP positive astrocytes can be imaged in three dimensions. Bitplane Imaris software can then be used to obtain measurements of the morphometric properties of astrocytes including surface area and volume. Synaptic colocalization of Lck-GFP expressing astrocytes can also be measured via immunohistochemistry for synaptic markers such as synapsin I and post-synaptic density 95 (PSD-95).

Using this approach, research in the Reissner lab has shown that following two weeks of ShA cocaine self-administration and 14-16 days of extinction training, astrocytes in the NAc show a significant decrease in surface area, volume, and synaptic colocalization with synaptic markers such as synapsin-I and PSD-95 (Scofield et al., 2016b; Testen et al., 2018). Further, these changes in NAc astrocytes are not observed 24 hours following ShA cocaine self-administration alone, suggesting that the changes in astrocyte morphology and decrease in synaptic colocalization requires a period of extinction or withdrawal (Testen et al., 2018).

Furthermore, these changes in astrocytes are specific to the NAc, as these alterations are not observed in the prefrontal cortex or basolateral amygdala (Testen et al., 2018). The results from these studies reveal that NAc astrocytes exist in an atrophic, retracted state following withdrawal from ShA cocaine self-administration. Furthermore, the decrease in synaptic colocalization of NAc astrocytes indicates that during withdrawal from cocaine use, the ability of astrocytes to modulate synaptic functions and synaptic plasticity may be reduced, which may contribute to the dysregulated synaptic function believed to contribute to increased relapse vulnerability.

Although these changes in NAc astrocytes have been well characterized after ShA cocaine self-administration and extinction training, it is currently unknown if these findings extend to other rodent models of drug use. In addition to the ShA/extinction model of rodent cocaine self-administration, the incubation of cocaine craving model has been widely used to model human drug use (Li et al., 2015; Lu et al., 2004). This model typically consists of longer access (LgA) to cocaine self-administration (6 hours/day) followed by prolonged abstinence (30 – 45 days) in the home cage. This paradigm leads to greater cocaine-seeking (i.e. an incubation of cocaine craving) across the duration of abstinence (Ferrario et al., 2005; Li et al., 2015), reaching a peak at ~45 days of abstinence, before beginning to decline at ~ 60 days (Lu et al., 2004; Pickens et al., 2011).

The incubation of cocaine craving is characterized by numerous synaptic changes within the NAc which drive potentiated drug seeking (Wolf, 2016). Interestingly, the downregulation of NAc GLT-1 protein expression is correlated with both the amount of cocaine self-administered, as well as the length of withdrawal (Fischer-Smith et al., 2012). Longer access to cocaine and longer abstinence periods lead to augmented decreases in GLT-

1 (Fischer-Smith et al., 2012). Moreover, preliminary data for this dissertation shows that LgA cocaine self-administration and prolonged abstinence leads to a downregulation in NAc GLT-1 mRNA levels, a phenomenon not observed following ShA/extinction (Kim et al., 2018b). These experiments suggest that compared to ShA/extinction, LgA cocaine self-administration and prolonged abstinence leads to intensified effects on NAc GLT-1 expression. Since GLT-1 is primarily expressed on astrocytes, I hypothesized that the decrease in morphometric properties and synaptic colocalization of NAc astrocytes might also be augmented (similarly as GLT-1 expression) following LgA cocaine self-administration and prolonged abstinence. This hypothesis is examined in experiment 3.1 (male rats) and in experiment 3.2 (female rats).

Methods

Experiment 3.1 (male rats) and experiment 3.2 (female rats): Effects of LgA cocaine self-administration and abstinence on the morphology and synaptic colocalization of NAc astrocytes

Animals

Male (225 – 250 g, experiment 3.1) and female (200 – 225 g, experiment 3.2) Sprague-Dawley rats, aged approximately 6-8 weeks were purchased from Envigo (Dublin, VA). All rats were housed individually in temperature and humidity controlled clear standard plexiglass cages on a reverse light-dark cycle (7 AM off, 7 PM on). All rats were allowed to acclimate to the animal facility for one week, where food and water were available ad libitum. Following the acclimation period, all rats were placed on a food restricted diet of ~ 20 g of chow per day. Food restriction lasted throughout all surgical, post-operative, and food-training procedures. Rats were then returned to an ab libitum diet which lasted throughout the duration of the study. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Surgical procedures

Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). Prior to the start of surgical procedures, all rats were administered an i.p. injection of the analgesic meloxicam (4 mg/kg). Silastic catheters with a 22-gauge cannula (0.02 mm inner diameter, 0.047 mm outer diameter) were implanted into the rats right jugular vein as previously described (Kim et al., 2018b; Sepulveda-Orengo et al., 2018). Immediately following jugular vein catheterization, rats were microinjected with lymphocyte protein kinase Lck-GFP, under the control of an astrocyte specific GfaABC1D promoter, packaged into the AAV5 serotype (6.1 x 10^{12} virus molecules/mL) by the UNC Viral Vector Core as previously described (Scofield et al., 2016a; Testen et al., 2019; Testen et al., 2018). Bilateral microinjections targeted the NAc (6° angle, AP +1.5, ML +2.6, DV -7.2), and virus was microinjected (0.1 µL/min, 1 µL per hemisphere) using 26-gauge guide cannulas (Plastics One, Roanoke, VA). Microinjectors were left in place for approximately 20 minutes to allow for virus diffusion and then slowly removed over a period of 1-2 minutes. Following surgical procedures, rats were administered an antibiotic (gentamicin 5 mg/mL, 0.1 mL) and heparinized saline (30 units/kg, 0.1 mL) intravenously through the implanted catheter for five days post-surgery, as well as throughout all self-administration procedures. Before the start of self-administration procedures, patency of the catheters was examined by administering a sub-threshold dose of propofol (10 mg/mL, 0.05 mL).

Behavioral Procedures

All food training and self-administration procedures were conducted in standard sound attenuated operant conditioning chambers (Med Associates, St. Albans VT). Prior to the start of self-administration, to facilitate acquisition to lever pressing, all rats were received one food

training session, where lever presses on the active lever resulted in the delivery of one 45 mg food pellet (Bio Serv, Flemington, NJ). Food training sessions lasted a minimum of 6 hours and criteria for food training was set at greater than 100 responses on the active lever. Following food training, rats were randomly assigned to two distinct groups, where one group lever pressed for cocaine and the other group for saline. As previously described (Kim et al., 2018b), all rats underwent LgA cocaine or saline self-administration for 6 hours/day on an FR1 schedule for 10 consecutive days. A response on the active lever resulted in the delivery of cocaine (0.75 mg/kg/infusion, both sexes) or saline (0.9% sodium chloride), along with the presentation of audio (70 dB, 2.5 kHz tone) and visual (stimulus light above the active lever) cues for five seconds. A response on the active lever was followed by a 20-sec time out period. Active lever presses during this time resulted in no programmed responses. Responses on the inactive lever at any time during the session were recorded but also resulted in no programmed responses. Following 10 days of LgA cocaine or saline self-administration, all rats underwent experimenter-induced abstinence in the home cage for 45 days. Throughout the 45 days of abstinence, all rats were handled at least two times per week.

Immunohistochemistry

For experiments 3.1 and 3.2, rats were euthanized 24 hours following the last day of abstinence. All rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 1x phosphate buffer (PB), followed by 4% paraformaldehyde (PFA, in 1x PB). Brains were extracted, post-fixed in 4% PFA for ~4 hours, and then stored in 30% sucrose. Tissues sections (100 μm thick) from the NAc were collected using a cryostat (Leica Biosystems, Buffalo Grove, IL) and stored in 50% glycerol/PBS until immunostaining.

For immunohistochemistry, free floating 100 µm NAc sections were first washed (3 x 5 min) in 1x PBS containing 2% Triton X-100 (PBST) (Thermo Fisher, Waltham, MA). Sections were then blocked in 5% normal goat serum (NGS, Sigma Aldrich, St. Louis, MO) in PBST (2%) for 1 hour at room temperature. Blocking solution was then replaced with primary antibodies (all at 1:500) in 5% NGS in PBST. The primary antibodies used in experiments 3.1 and 3.2 were mouse anti-PSD-95 (Thermo Fisher, Waltham, MA) and rabbit anti-GFAP (Dako, Santa Clara, CA). Sections were probed with primary antibodies for 72 hours at 4 °C. Slices were flipped halfway through the incubation period to allow maximum penetration of primary antibodies. Following incubation for primary antibodies, slices were first washed (3 x 5 min) in PBST and transferred to new wells. Secondary antibodies were added to a solution containing 5% NGS in PBST. Identical to the incubation period for primary antibodies, slices were probed with secondary antibodies for 72 hours at 4 °C. Secondary antibodies used (all at 1:1000) were goat anti-mouse Alexa Fluor 594 (Thermo Fisher, Waltham, MA) and goat anti-rabbit Alexa Fluor 647 (Thermo Fisher, Waltham, MA). Following incubation with secondary antibodies, sections were washed 3 x 10 min in PBST (0.2%), followed by one wash in 1x PBS. Sections were stored in 1x PBS at 4 °C until image acquisition.

Astrocyte Image Acquisition and Processing

Image acquisition and processing of NAc astrocytes were identical to methods described previously (Testen et al., 2019; Testen et al., 2018). Sections were mounted onto slides and cover slipped immediately with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL). A Zeiss LSM 800 confocal-scanning microscope (405/488/561/640 nm diode lasers, 2 GaAsP detectors, 63x oil-immersed objective), was used with the following parameters for image acquisition: 1024 x 1024 pixels, bit depth 16-bit, 4x averaging, 1µm z-

step. Only single, isolated astrocytes within the NAc were acquired. Astrocytes were not imaged if they were outside of the NAc, bordering and near proximity of other astrocytes, or cut within the z-plane during sectioning.

Following image acquisition, raw images were deconvolved using AutoQuant software (v. X3.0.4, MediaCybernetics) and imported into Imaris software (v 8.4.1, Bitplane, Zurich, Switzerland) for analysis. Using the Lck-GFP fluorescence from each astrocyte, each cell was reconstructed in 3-dimensions and a surface was built to obtain measurements of surface area and volume. A masked channel was then created to isolate the astrocyte Lck-GFP signal from background, and this masked channel was then used to perform colocalization analysis between the masked Lck-GFP signal and PSD-95, represented by the Alexa 594 signal. A colocalization channel was then generated to obtain the percentage of masked Lck-GFP signal (defined as the region of interest, ROI) colocalized with PSD-95. For PSD-95 analysis, a 50 µm x 50 µm x 50 µm box was constructed around the isolated astrocyte, and the number of positive PSD-95 puncta above a predetermined threshold was automatically counted in Bitplane Imaris. All imaging and analysis were done blind to groups.

Data Analysis

All statistical analysis for behavioral data was conducted using SigmaPlot (v.11) or SPSS (v. 25) software. For all behavioral measures, a mixed ANOVA ($\alpha = 0.05$) was performed with drug (saline vs. cocaine) and time (self-administration session) set as independent variables. The dependent variable for behavioral measures was active lever presses or infusions received during self-administration. For all astrocyte imaging data, SAS (v. 9.4) software was used to perform a nested ANOVA (Heimer-McGinn et al., 2013; Risher et al., 2014; Testen et al., 2018) comparing saline vs. cocaine self-administering rats, with

surface area, volume, synaptic colocalization, or the number of PSD-95 puncta above threshold set as dependent variables. To compare behavioral data across sex, a mixed ANOVA ($\alpha = 0.05$) was performed with sex (male vs. female), drug (saline vs. cocaine) and time (self-administration session) set as independent variables. The dependent variable was the number of active lever presses or infusions received. To compare astrocyte morphology and synaptic colocalization data across sex, a nested ANOVA was performed comparing male vs. female saline self-administering rats with surface area, volume, synaptic colocalization, or the number of PSD-95 puncta above threshold set as dependent variables.

Experiment 3.2: Effects of LgA cocaine self-administration and abstinence on the morphology and synaptic colocalization of NAc astrocytes in female rats

Cytological Assessment to Determine Estrous Cycle Phase in Female Rats

All surgical, behavioral, immunohistochemistry and confocal imaging procedures were identical between experiments 3.1 and 3.2. However, in experiment 3.2, the estrous cycle stage for each female rat was determined at the time of euthanasia. The procedures used to determined estrous cycle were identical to the methods used in Chapter 2, and also outlined in a previous study (Marcondes et al., 2002). Briefly, a sterile swab moistened with saline was used to smear for phases of the estrous cycle. The smear was placed along a glass slide which was then stained using 1 mL of Wright's stain. The slices were then evaluated under 10x objective light microscope. Using previously defined criteria (Marcondes et al., 2002), the estrous cycle phase (metestrus, diestrus, estrus or proestrus) for each rat was determined. Similar to previous experiments (Bechard et al., 2018; Calipari et al., 2017), female rats in the metestrus and diestrus stages were both characterized as having low amounts of circulating hormone levels and thus, were combined into one group. The proestrus stage is characterized

by elevated levels of circulating hormones, including increased levels of luteinizing hormone, follicle-stimulating hormone, progesterone and 17β- estradiol. Likewise, the estrus stage is also characterized by increased levels of sex hormones, and is the stage at which ovulation occurs.

Results

Experiment 3.1: Effects of LgA cocaine self-administration and prolonged abstinence on the morphology and synaptic colocalization of NAc astrocytes in male rats

Single-cell analysis of NAc astrocytes using AAV5-GfaABC1D-Lck-GFP is shown in Figures 3.1. Only single, isolated astrocytes were selected for analysis (Fig 3.1a far left, 20x magnification). To verify that image analysis was specific to astrocytes, all slices were probed for glial fibrillary acidic protein (GFAP), an astrocyte-specific marker (Fig 3.1a second panel (20x) and Fig 3.1b (63x)), as well as for the nucleic acid marker DAPI (Fig 3.1a third panel, 20 x image). Only astrocytes within the NAc were analyzed (Fig 3.1c). Any cells outside of the NAc were excluded from analysis.

Behavioral data for all male rats used in the NAc astrocyte imaging experiment are shown in Figures 3.2a and 3.2b. Compared to saline self-administering rats, cocaine self-administering rats exhibited a significantly greater amount of both active lever presses ($F_{(1, 199)} = 172$, $p < 0.001$; Fig 3.2a) and number of infusions received ($F_{(1, 199)} = 205.33$, $p < 0.001$; Fig 3.2b). For both active lever presses ($F_{(9, 199)} = 10.14$, $p < 0.001$) and infusions ($F_{(1, 199)} = 9.68$, $p < 0.001$), the main effect of time was also significant. For infusions, the interaction between drug and time was also significant ($F_{(9, 199)} = 16.63$, $p < 0.001$; Fig 3.2b). Pairwise comparisons using the Holm-Sidak method showed that cocaine self-administering rats showed a significantly greater amount of infusions received on day 9 (vs. day 1 $t = 3.68$, $p <$

0.001; vs. day 2 $t = 4.27$, $p < 0.001$) and day 10 (vs. day 1 $t = 4.41$, $p < 0.001$; vs. day 2 $t = 5.00$, $p < 0.001$), indicating an escalation of cocaine intake, which was not seen in saline-self-administering rats.

Following 45 days of abstinence from saline or cocaine self-administration, Bitplane IMARIS software was used to reconstruct a surface around isolated NAc astrocytes to obtain morphometric data (Fig 3.2c). Compared to astrocytes in the NAc of saline self-administering male rats, NAc astrocytes in male rats following 45 days of abstinence from LgA cocaine self-administration showed a significant decrease in both surface area ($F_{(1, 18)} = 50.51$, $p < 0.001$; Fig 3.2d) and volume ($F_{(1, 18)} = 21.76$, $p < 0.05$; Fig 3.2e).

To assess synaptic colocalization of NAc astrocytes in male rats, colocalization between the masked Lck-GFP channel and the post-synaptic marker PSD-95 was used to generate a new colocalization channel (Fig 3.3a). After 45 days of abstinence following LgA cocaine self-administration, the colocalization between Lck-GFP and PSD-95 positive voxels was significantly lower than colocalization between the two channels in rats that had previously self-administered only saline ($F_{(1, 18)} = 30.2$, $p < 0.001$; Fig 3.3b). This difference in synaptic colocalization was not due to an effect of cocaine on PSD-95, as there was no statistically significant difference saline and cocaine self-administering rats in the number of PSD-95 positive puncta above threshold ($F_{(1, 18)} = 1.53$, $p = 0.23$; Fig 3.3c).

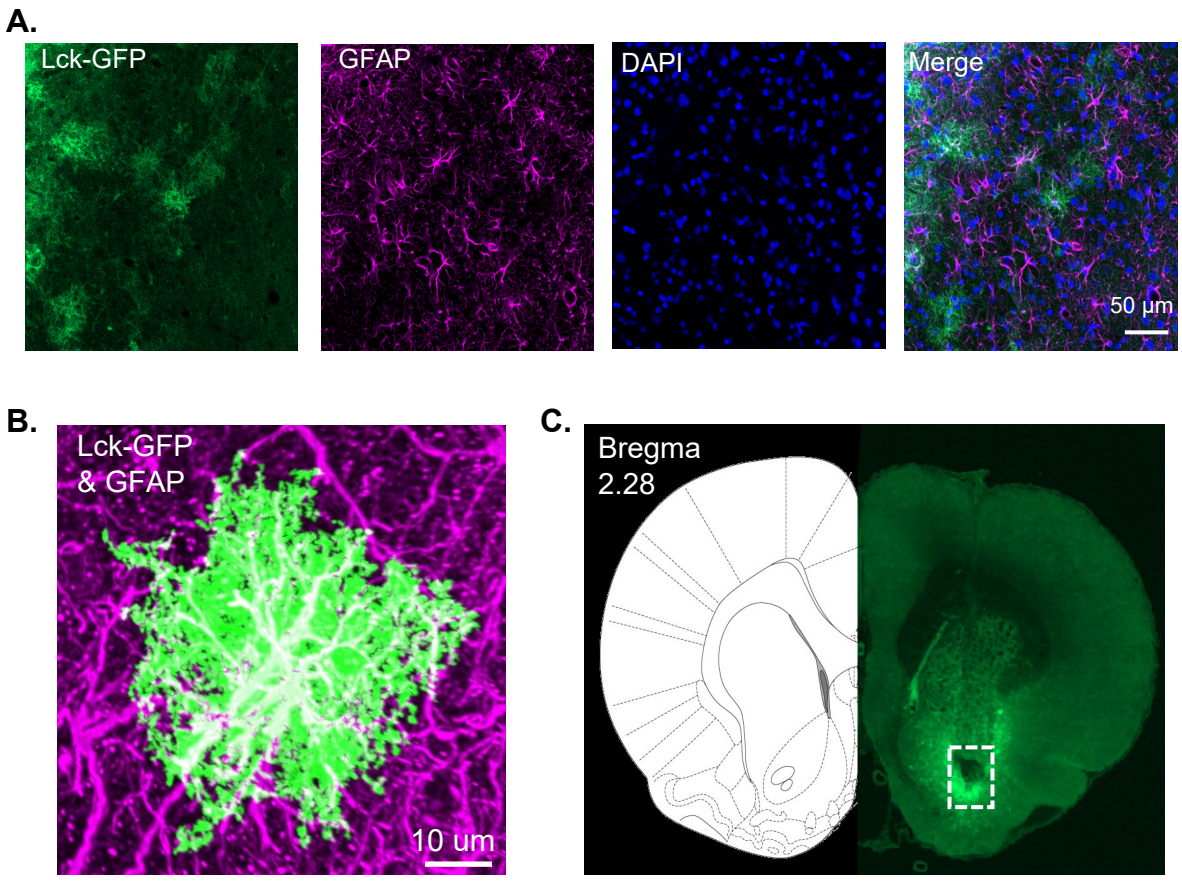

Figure 3.1 Single cell analysis of NAc astrocytes using AAV5-GfaABC1D-Lck-GFP. (A) 20x confocal images of the NAc containing: Lck-GFP expressing astrocytes (far left), immunohistochemistry for the cytoskeletal astrocyte marker GFAP (second panel), stain for the DNA marker DAPI (third panel), and a merge of all three channels (far right) **(B)** 63x confocal image of a single, isolated Lck-GFP expressing astrocyte (green), along with GFAP (purple), and the overlay between the two (white). Lck-GFP was expressed throughout the entire membrane of the astrocyte, including peripheral astrocytic processes (PAP's), which are unable to be visualized using traditional GFAP staining. **(C)** Scan of a whole brain slice containing the NAc, demonstrating intensity and spread of AAV5-Lck-GFP. Only astrocytes within the white square were selected for further analysis

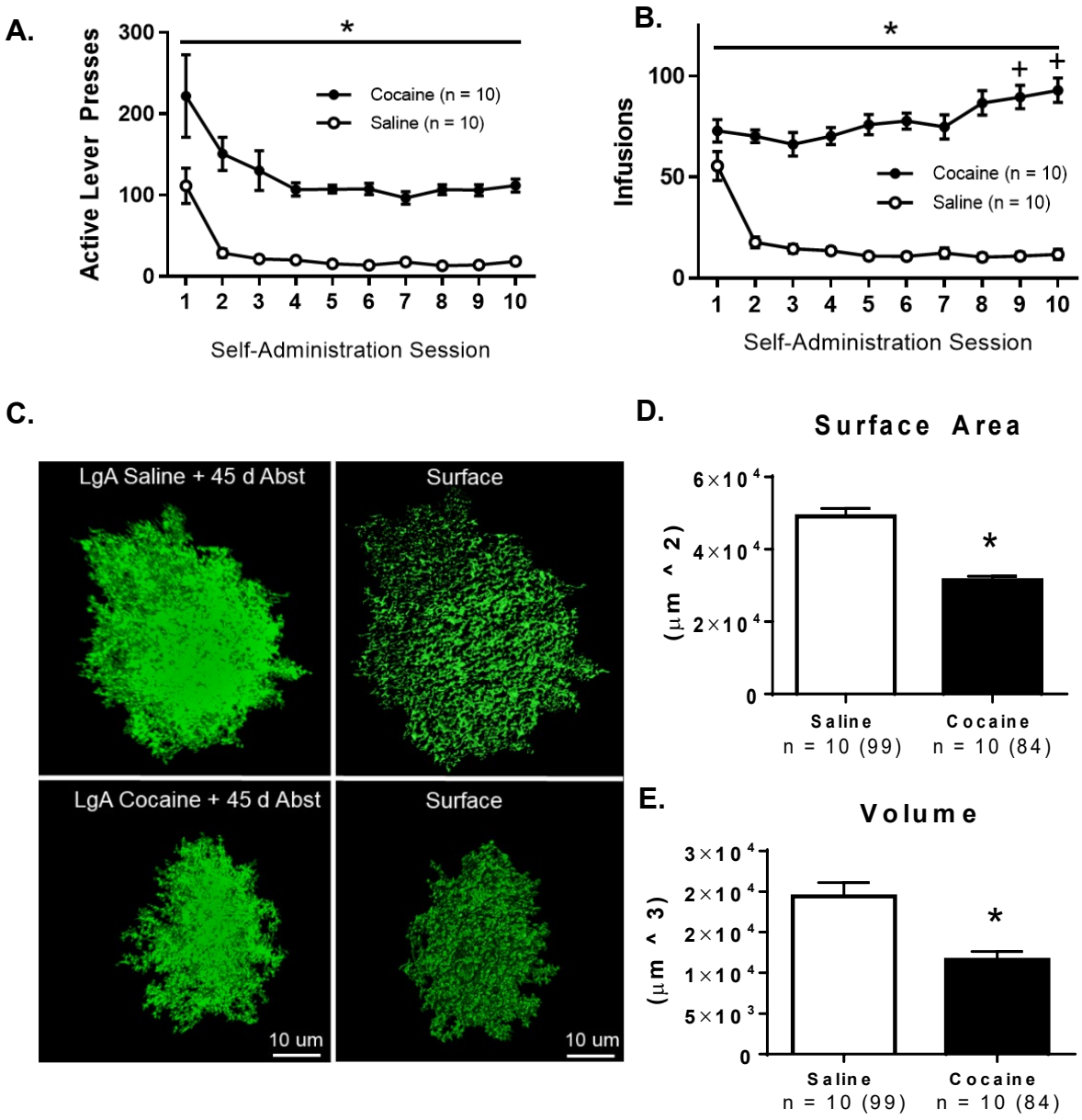

Figure 3.2 Behavioral data for all male rats and morphometric analysis of NAc astrocytes following LgA cocaine self-administration and prolonged abstinence in male rats. (A) active lever presses and **(B)** infusions received during self-administration. Cocaine self-administering male rats showed an increase in both active lever presses and infusions received vs. saline self-administering male rats (* p <0.001), and also exhibited an escalation of cocaine intake (+ p <0.001) **(C)** 63x confocal image of a single, isolated Lck-GFP expressing astrocyte in the NAc (left panels), along with surface reconstruction of isolated astrocytes (right panels). **(D)** Surface area and **(E)** volume of NAc astrocytes was significantly decreased following LgA cocaine self-administration and prolonged abstinence in male rats (* p < 0.05).

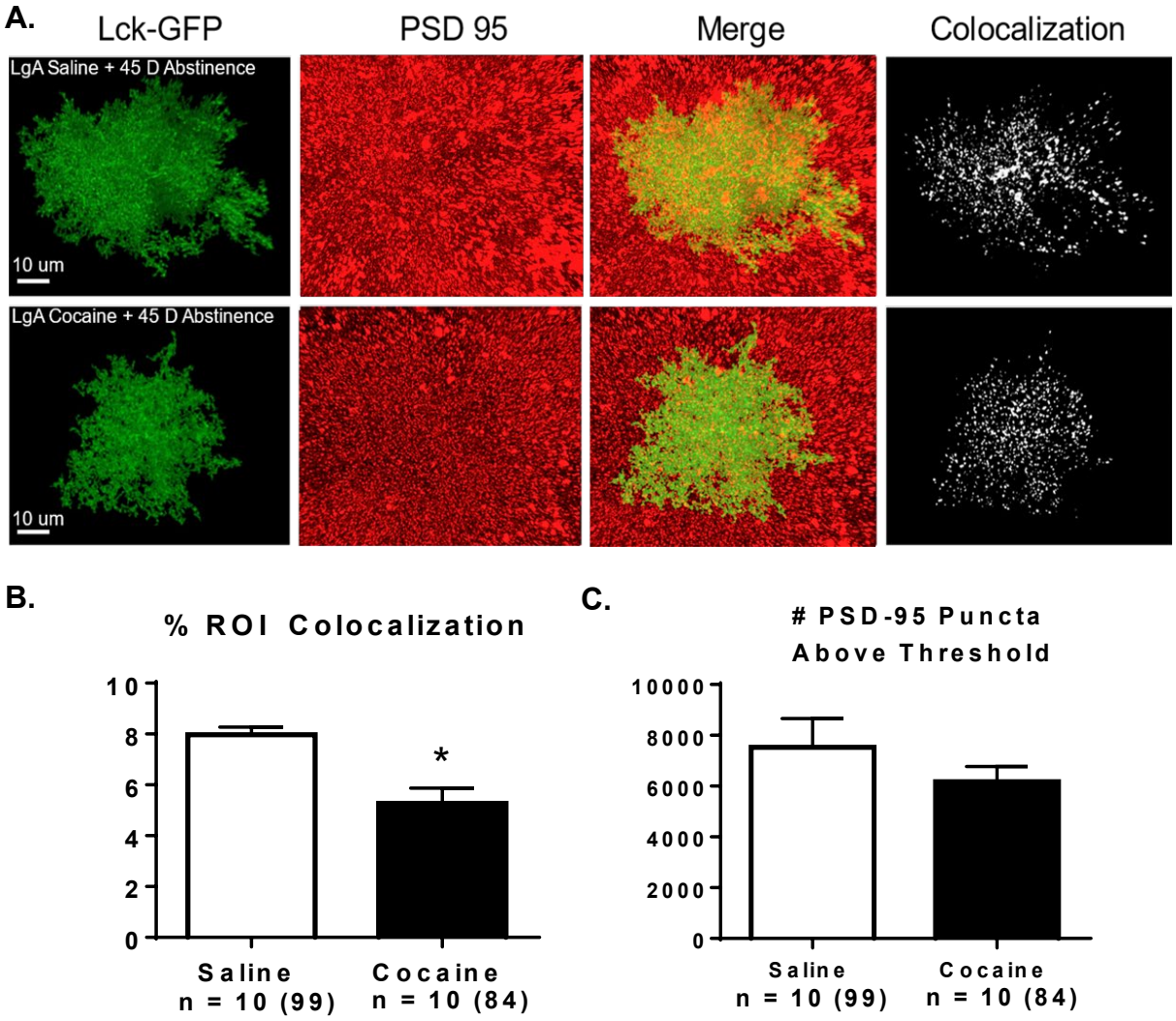

Figure 3.3 Synaptic colocalization of NAc astrocytes after LgA cocaine self-administration and prolonged abstinence in male rats. (A) 63x confocal image of an isolated Lck-GFP expressing astrocyte in the NAc (far left), immunohistochemistry for the synaptic marker PSD-95 (second panels), merge of Lck-GFP and PSD-95 channels (third panels), and colocalization of Lck-GFP expressing astrocytes with PSD-95 (far right). **(B)** Synaptic colocalization of NAc astrocytes was significantly decreased after LgA cocaine self-administration and prolonged abstinence in male rats (* $p < 0.05$). **(C)** The number of positive PSD-95 puncta above threshold was not significantly different between saline and cocaine self-administering male rats.

Experiment 3.2: Effects of LgA cocaine self-administration and prolonged abstinence on the morphology and synaptic colocalization of NAc astrocytes in female rats

Behavioral data for all female rats used in experiment 3.2 is shown in Figures 3.4a and 3.4b. Cocaine self-administering female rats showed a significantly greater number of both active lever presses ($F_{(1, 239)} = 54.45$, $p < 0.001$; Fig 3.4a) and number of infusions received (F (1, 239) = 190.59, $p < 0.001$; Fig 3.4b) in comparison to saline self-administering female rats. For both active lever presses ($F_{(9, 239)} = 11.03$, $p < 0.001$; Fig 3.4a) and infusions ($F_{(9, 239)} = 4.55$, $p < 0.001$; Fig 3.4b) the main effect of time was also significant. For infusions, the interaction between drug and time was also significant ($F_{(9, 239)} = 28.56$, $p < 0.001$; fig 3.4b). Pairwise comparisons using the Holm-Sidak method showed that cocaine self-administering female rats showed a significantly greater amount of infusions received on day 9 (vs. day 1 $t = 6.98$, $p < 0.001$; vs. day 2 $t = 7.17$, $p < 0.001$) and day 10 (vs. day 1 $t = 6.13$, $p < 0.001$; vs. day 2 $t = 6.33$, $p < 0.001$) of cocaine self-administration (Fig 3.4b), indicating an escalation of cocaine intake, which was not seen in saline-self-administering female rats.

Single-cell analysis of NAc astrocytes in female rats was completed identically to the procedures outlined to assess the morphometric properties of NAc astrocytes in male rats. Although astrocytes from the NAc of male rats exhibited a pronounced decrease in both surface area and volume following LgA cocaine self-administration and 45 days of abstinence (Fig 3.2c-e), this decrease was not seen in NAc astrocytes from female rats (Fig 3.4c-e). There was no significant difference in surface area ($F_{(1, 22)} = 0.81$, $p = 0.38$; Fig 3.4d) or volume ($F_{(1, 22)} = 1.83$, $p = 0.19$; Fig 3.4e) between saline and cocaine self-administering female rats following 45 days of abstinence. To examine a possible role of the estrous cycle in modulating these results, saline and cocaine self-administering female rats were further divided into distinct

groups based on estrous cycle stage. When the estrous cycle was accounted for, there was no effect of drug (cocaine vs. saline ($F_{(1, 18)} = 1.35$, $p = 0.26$; Fig 3.5a) or estrous cycle stage ($F_{(2, 18)} = 1.07$, $p = 0.36$; Fig 3.5a) on the surface area of NAc astrocytes. The interaction between drug and estrous cycle stage was also not significant $F_{(2, 18)} = 0.34$, $p = 0.71$; Fig 3.5a). Furthermore, there was no effect of drug (cocaine vs. saline ($F_{(1, 18)} = 0.79$, $p = 0.39$; Fig 3.5b) or estrous cycle stage ($F_{(2, 18)} = 0.71$, $p = 0.50$; Fig 3.5b) on the volume of NAc astrocytes. The interaction between drug and estrous cycle stage was also not significant ($F_{(2, 18)} = 0.22$, $p = 0.81$; Fig 3.5b).

Analysis of synaptic colocalization of NAc astrocytes with the synaptic marker PSD-95 was completed in female rats identically to the procedures outlined in experiment 3.1 for male rats (Fig 3.6a). Although NAc astrocytes from male rats show a significant decrease in synaptic colocalization following LgA cocaine self-administration and prolonged abstinence (Fig 3.3a-b), there was no significant difference in synaptic colocalization of NAc astrocytes observed between saline and cocaine self-administering female rats ($F_{(1, 22)} = 2.24$, $p = 0.15$; Fig 3.6b). There was also no effect of cocaine on PSD-95 in female rats, as the number of PSD-95 positive puncta above threshold was not significantly different between groups ($F_{(1, 22)} = 0.04$, $p = 0.84$; Fig 3.6c) To examine a possible role of the estrous cycle in modulating these results, saline and cocaine self-administering female rats were further divided into distinct groups based on estrous cycle phase. When the estrous cycle was accounted for, there was no effect of drug (cocaine vs. saline ($F_{(1, 18)} = 0.01$, $p = 0.93$; Fig 3.8) or estrous cycle stage ($F_{(2, 18)} = 0.06$, $p = 0.94$; Fig 3.7) on synaptic colocalization of NAc astrocytes. The interaction between drug and estrous cycle stage was also not statistically significant ($F_{(2, 18)} = 0.56$, $p = 0.58$; Fig 3.7).

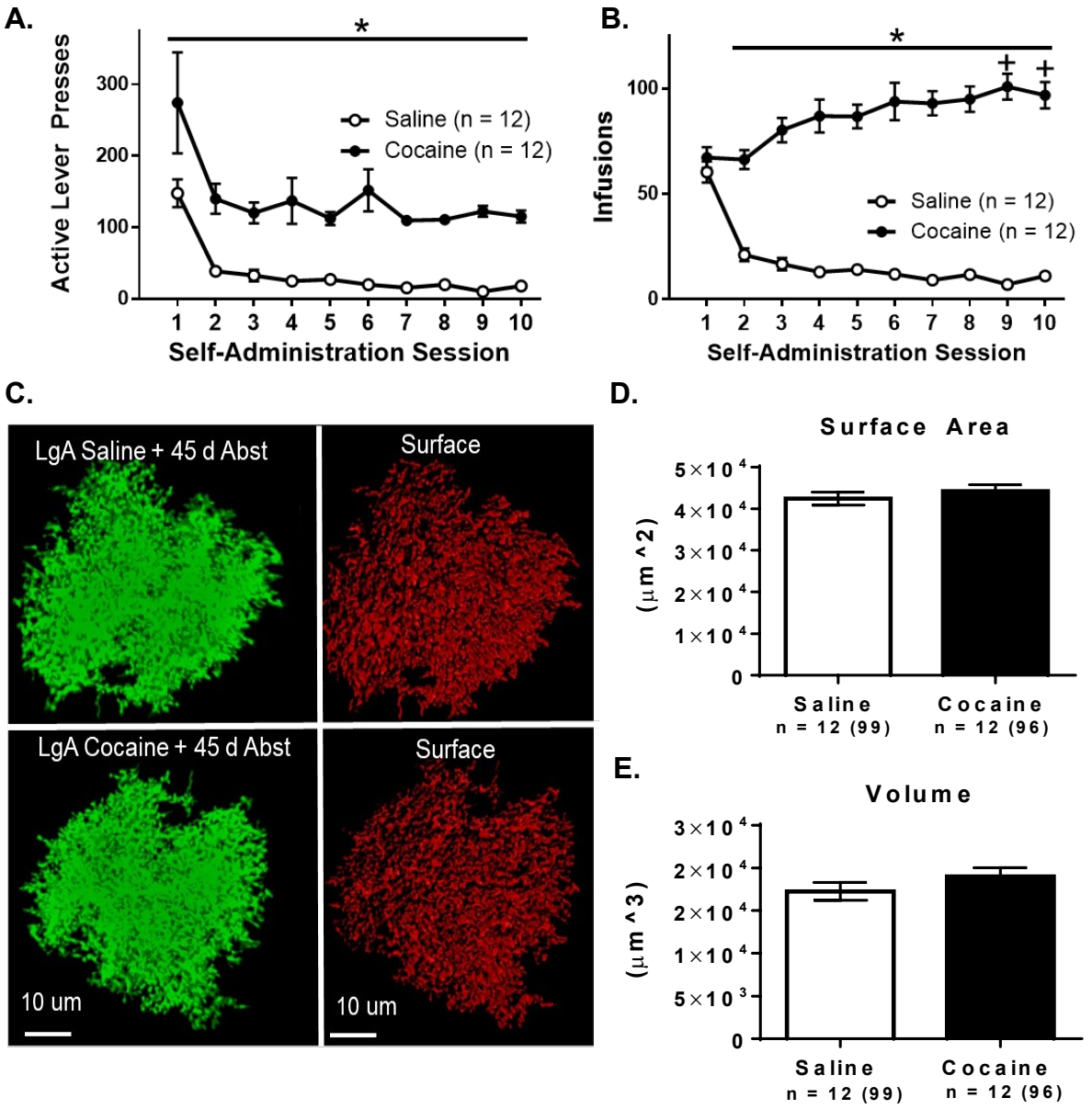

Figure 3.4 Behavioral data and morphometric analysis of NAc astrocytes for all female rats. (A) active lever presses and **(B)** infusions received during self-administration. Female rats lever pressing for cocaine showed a significant increase in the number of active lever presses and infusions vs. saline self-administering female rats (* $p < 0.001$). Female rats lever pressing for cocaine also exhibited an escalation of cocaine intake, with an increase in the number of infusions received on days 9 and 10 of self-administration (vs. days 1 and 2; + $p < 0.001$). **(C)** 63x confocal image of a single, isolated Lck-GFP expressing astrocyte in the NAc (left panels), along with surface reconstruction of isolated astrocytes (right panels). **(D)** Surface area and **(E)** volume of NAc astrocytes was unchanged following LgA cocaine self-administration and prolonged abstinence in female rats.

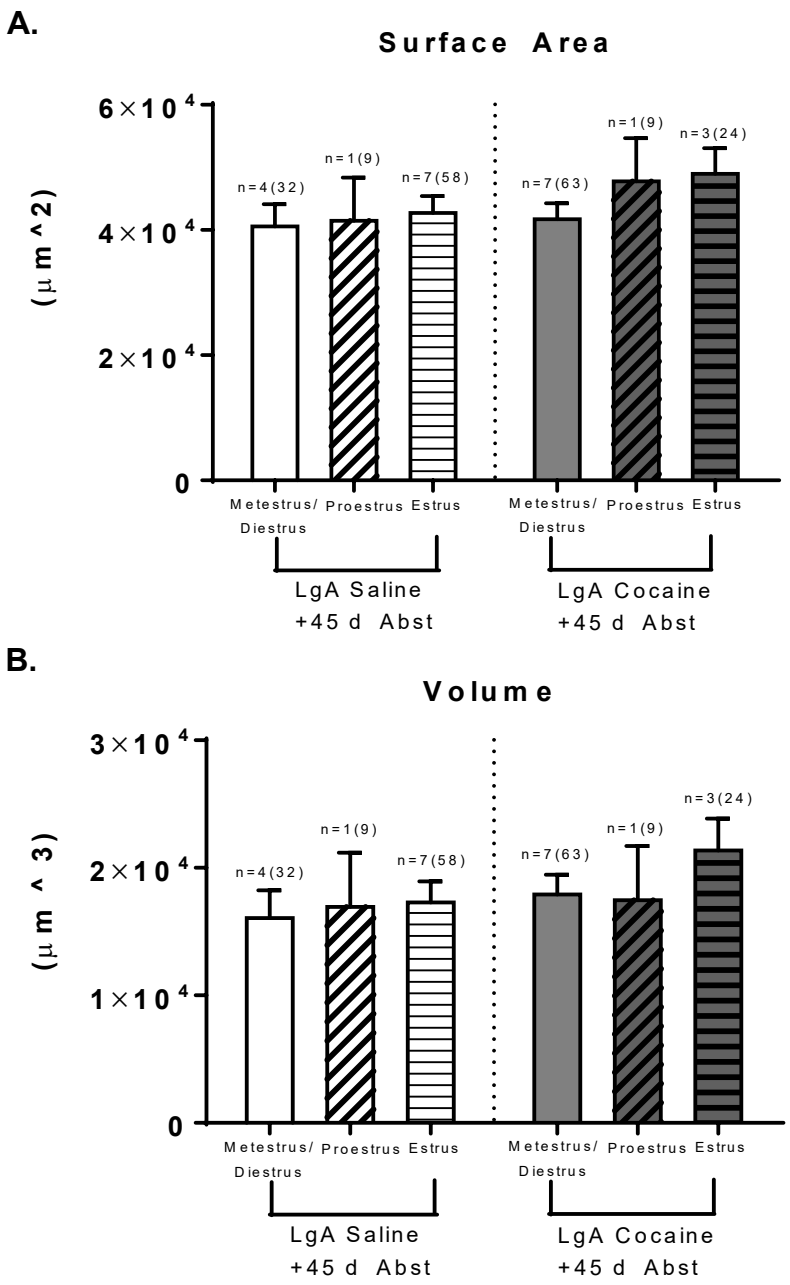

Figure 3.5 Morphometric analysis of NAc astrocytes following LgA cocaine self-administration and prolonged abstinence in female rats divided into distinct groups based on estrous cycle stage. There was no main effect of drug or estrous cycle stage on **(A)** surface area or **(B)** volume of NAc astrocytes. The interaction between drug and estrous cycle stage was also not significant.

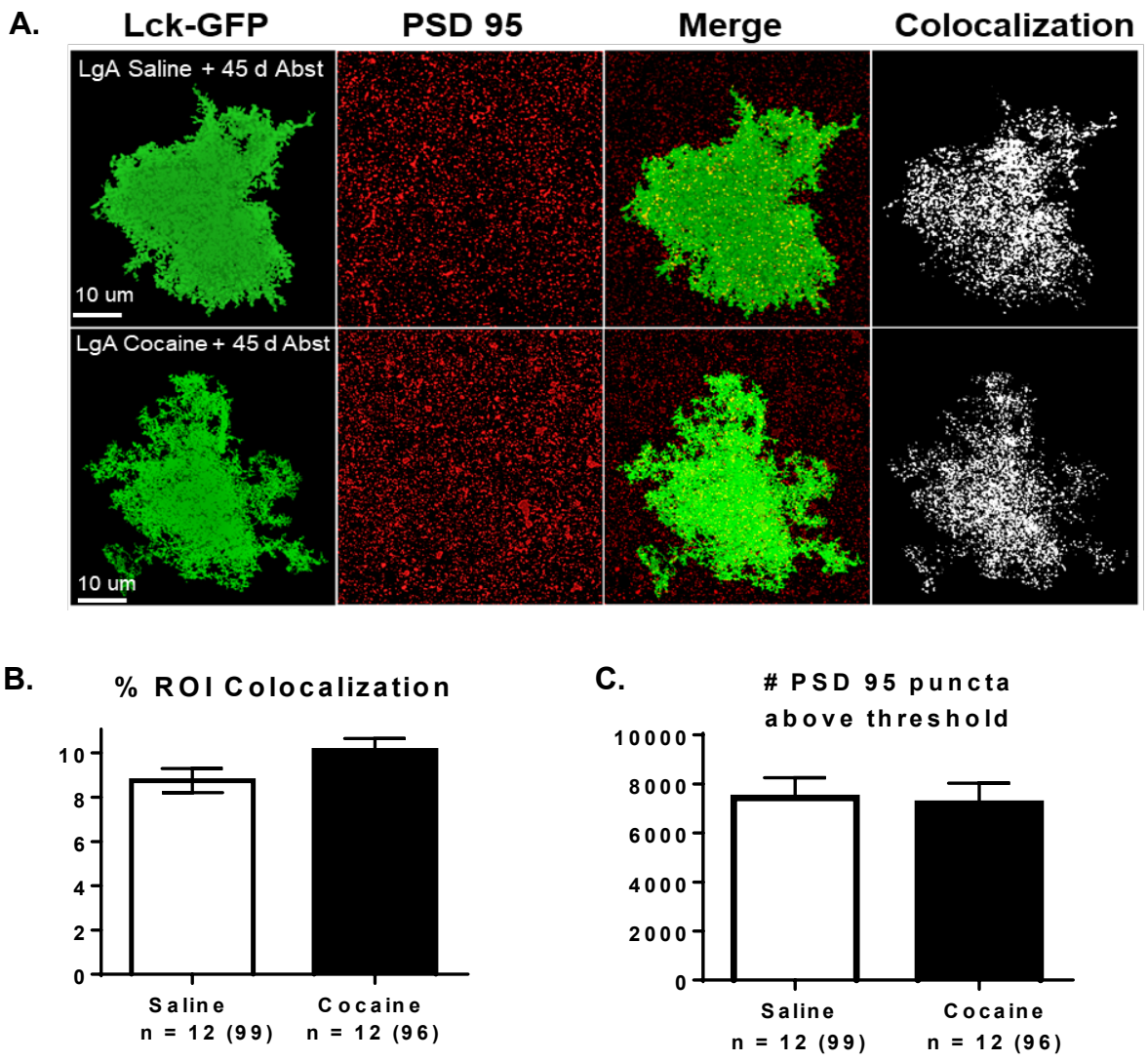

Figure 3.6 Synaptic colocalization of NAc astrocytes following LgA cocaine self-administration and prolonged abstinence in female rats. (A) 63x confocal image of an isolated Lck-GFP expressing astrocyte in the NAc (far left), immunohistochemistry for the synaptic marker PSD-95 (second panels), merge of Lck-GFP and PSD-95 channels (third panels), and colocalization of Lck-GFP expressing astrocytes with PSD-95 (far right). **(B)** There was no difference between saline and cocaine self-administering female rats in synaptic colocalization of NAc astrocytes **(C)** There was no difference between saline and cocaine self-administering female rats in the number of positive PSD-95 puncta above threshold.

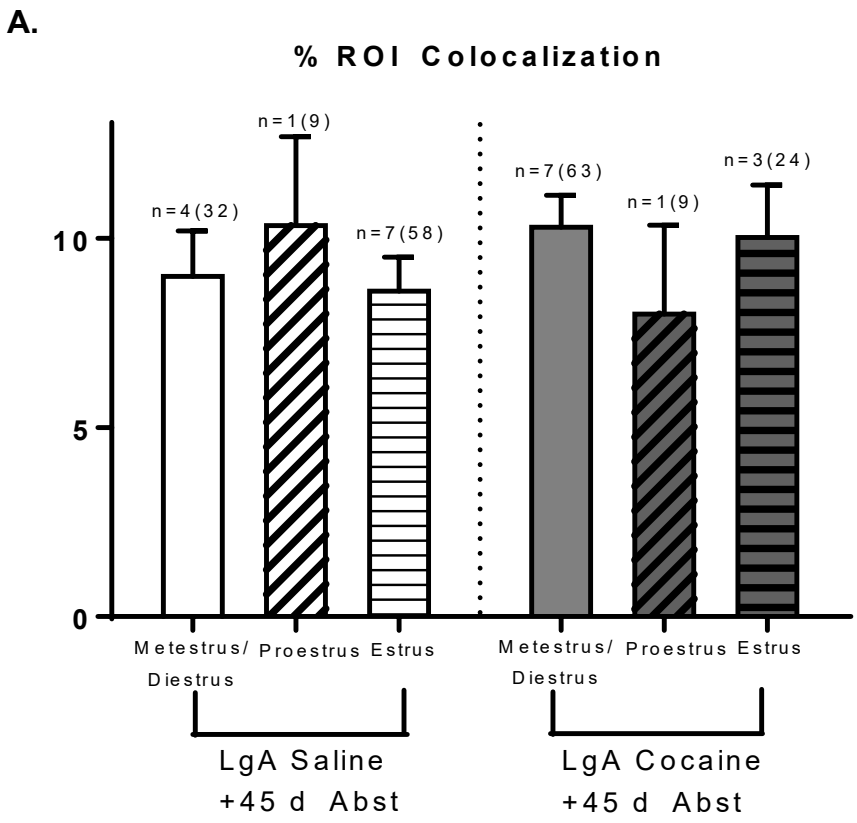

Figure 3.7 Synaptic colocalization of NAc astrocytes following LgA cocaine self-administration and prolonged abstinence in female rats divided into distinct groups based on estrous cycle stage. There was no main effect of drug or estrous cycle stage on **(A)** surface or **(B)** volume of NAc astrocytes. The interaction between drug and estrous cycle stage was also not significant.

Comparing astrocytes from male vs. female saline self-administering rats

Behaviorally, regardless of sex, rats lever pressing for cocaine exhibited a significant increase in both active lever presses ($F_{(1, 40)} = 141.62$, $p < 0.001$; Fig 3.8a) and infusions received ($F_{(1, 40)} = 373.7$, $p < 0.001$; Fig 3.8b) in comparison to rats lever pressing for saline. However, the main effect of sex was not statistically significant, as male and female rats did not differ in the number of active lever presses ($F_{(1, 40)} = 1.796$, $p = 0.188$; Fig 3.8a) or infusions received ($F_{(1, 40)} = 2.002$, $p = 0.165$; Fig 3.8b). Furthermore, for both active lever presses ($F_{(1, 40)} = 0.166$, $p = 0.686$) and infusions ($F_{(1, 40)} = 1.499$, $p = 0.228$) the interaction between sex and drug was not statistically significant. Within cocaine self-administering rats only, there was no significant difference between male and female rats in the number of active lever presses ($F_{(9, 219)} = 0.845$, $p = 0.369$) or infusions ($F_{(9, 219)} = 2.01$, $p = 0.172$). However, for infusions, the interaction between sex and self-administration session was statistically significant ($F_{(9, 219)} = 2.07$, $p < 0.05$; Fig 3.8b). Post-hoc tests using the Holm-Sidak method reveal that on days 4 ($t = 1.963$, $p < 0.05$), 6 ($t = 1.90$, $p < 0.05$) and 7 ($t = 2.141$, $p < 0.05$) of cocaine self-administration, female rats received a significantly greater number of cocaine infusions than male rats.

To better understand the sex difference in the changes in NAc astrocytes following abstinence from LgA cocaine self-administration, the morphometric changes and synaptic colocalization of NAc astrocytes between male and female saline self-administering rats were examined. Astrocytes from the NAc of saline self-administering female rats exhibited a significantly smaller surface area ($F_{(1, 20)} = 10.79$, $p < 0.01$; Fig 3.9a), but not volume ($F_{(1, 20)} = 2.63$, $p = 0.12$; Fig 3.9b), compared to astrocytes from the NAc of saline self-administering male rats. Furthermore, within saline self-administering rats, the synaptic colocalization ($F_{(1,}$

$_{20)}$ = 3.13, p < 0.092; Fig 3.10a), as well as the number of positive PSD-95 puncta ($F_{(1, 20)}$ = 0.01, p = 0.909; Fig 3.10b), were not statistically different between astrocytes from the NAc of male vs. female rats.

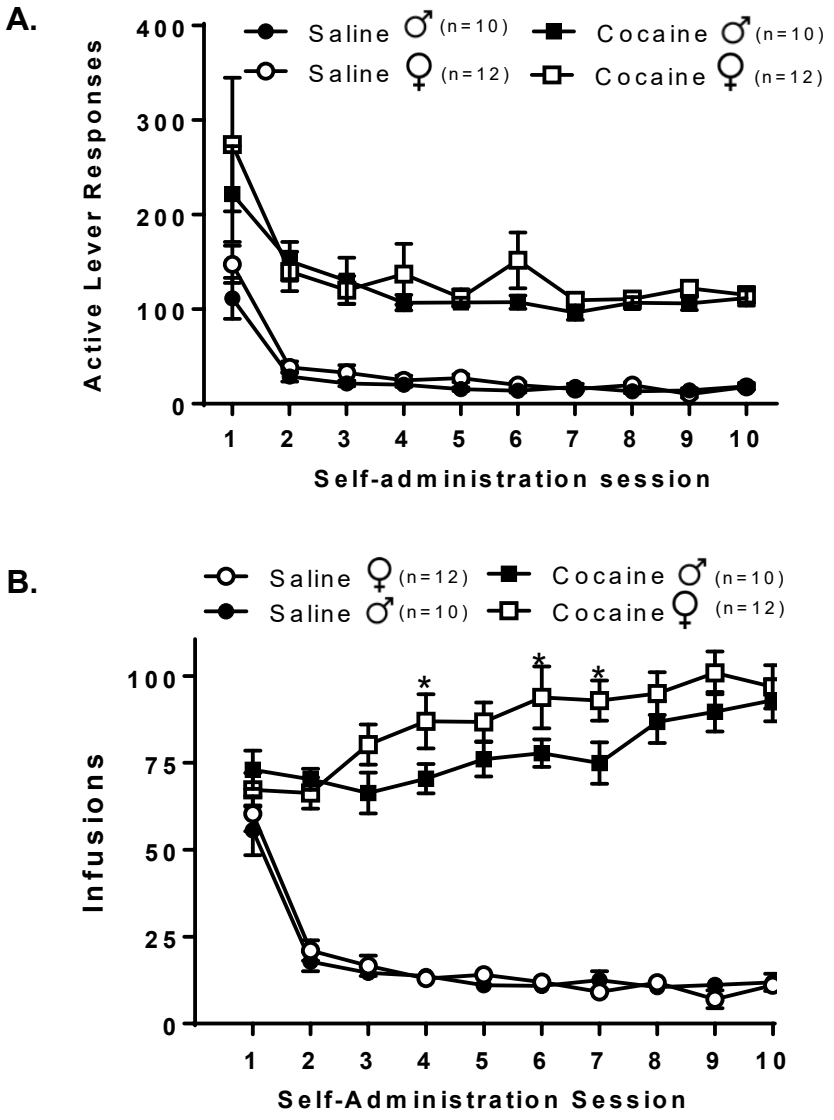

Figure 3.8 Comparing cocaine self-administration data between male and female rats. There was no main effect of sex on either **(A)** active lever presses or **(B)** infusions received during self-administration. However, when comparing the number of infusions received only within cocaine self-administering rats, female rats received a greater number of cocaine infusions on days 4, 6 and 7 of cocaine self-administration (* $p < 0.05$).

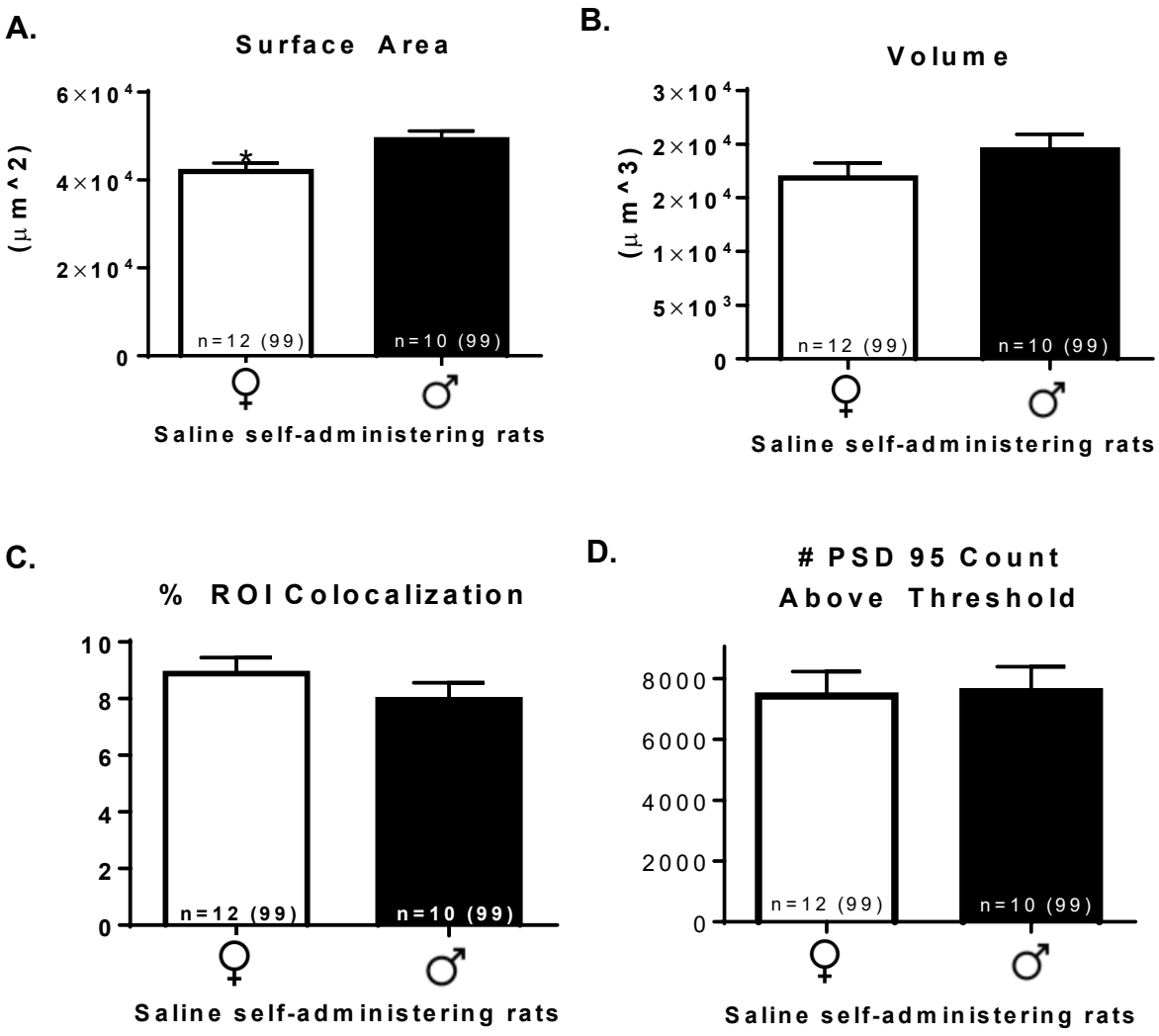

Figure 3.9 Comparing the morphometric properties and synaptic colocalization of NAc astrocytes in saline self-administering male vs. female rats. Within saline self-administering rats, astrocytes from the NAc of female rats exhibited a significant decrease (* $p < 0.05$) in **(A)** surface but not **(B)** volume in comparison to astrocytes from the NAc of male rats. Furthermore, there was no difference in synaptic colocalization or the number of PSD-95 puncta above threshold.

Discussion

The results from experiment 3.1 show that similar to previous experiments after ShA cocaine self-administration and extinction training (Scofield et al., 2016b; Testen et al., 2018), following 45 days of abstinence from LgA cocaine self-administration, astrocytes from the NAc of male rats exhibited a marked decrease in surface area, volume, and synaptic colocalization. The results from experiment 3.2 revealed that these changes in astrocytes were not observed in the NAc of female rats. These two experiments indicate a sex difference in the cocaine-induced changes in NAc astrocytes. However, results from experiment 3.2 indicate that in female rats, other mechanisms and cellular adaptations likely contribute to the incubation of cocaine craving.

The morphometric properties and synaptic colocalization of NAc astrocytes are decreased in male rats following abstinence from LgA cocaine self-administration

As mentioned above, previous studies have revealed that astrocytes from the NAc of male rats exhibit a decrease in surface area, volume, and synaptic colocalization after ShA cocaine self-administration and extinction training (Scofield et al., 2016b; Testen et al., 2018). The results from experiment 3.1 add to these findings and show similar, and perhaps even more pronounced decreases in the morphometric properties and synaptic colocalization of NAc astrocytes following prolonged abstinence from LgA cocaine self-administration. Although not directly comparable across the different studies, the results from this experiment suggest that in comparison to ShA/extinction, LgA cocaine self-administration and prolonged abstinence likely leads to greater effects on the morphology and synaptic colocalization of NAc astrocytes. This result extends findings from previous studies which have shown that in comparison to ShA/extinction, LgA cocaine self-administration and prolonged abstinence

leads to greater effects on cocaine-seeking (Li et al., 2015; Lu et al., 2004), synaptic changes in the NAc (Loweth et al., 2014; Wolf, 2016), and NAc GLT-1 expression (Fischer-Smith et al., 2012; Kim et al., 2018b).

Since the morphometric changes in NAc astrocytes have been well established following multiple different cocaine self-administration paradigms, the next logical step is to examine the mechanism underlying this retracted phenotype. Interestingly, astrocytes also show a decrease in morphometric features following chronic stress and anxiety, a finding that has been observed and reported widely (Kim et al., 2018a). For example, the somal volume of astrocytes is decreased in the hippocampus of male tree shrews following psychosocial stress (Czeh et al., 2006). In rodents, chronic stress has been shown to lead to a decrease in the number of astrocytic primary processes in the hippocampus (Saur et al., 2016), as well as a decrease in the branching, length and volume of primary processes in the frontal cortex (Tynan et al., 2013). In support of this hypothesis astrocytes from human postmortem brains of depressed patients also show decreased GFAP expression (Altshuler et al., 2010; Chandley et al., 2013; Cobb et al., 2016; Si et al., 2004; Webster et al., 2001).

One way in which stress can induce changes in astrocyte morphology is via activation of the glucocorticoid receptor. For example, stimulation of glucocorticoid receptors has been shown to decrease the number of astrocytes in the hippocampus (Lou et al., 2018; Unemura et al., 2012; Yu et al., 2011). Since it is well established that stress levels are increased during withdrawal from drug use (Alves et al., 2014; Garcia-Fuster et al., 2012), a similar mechanism may also be inducing changes in astrocyte morphology and synaptic colocalization during abstinence from LgA cocaine self-administration. Future experiments can examine this hypothesis by examining corticosterone levels during abstinence. Another potential

experiment can use agonists or antagonists at the glucocorticoid receptor to directly examine the relationship between glucocorticoids, activation of the glucocorticoid receptor, and the cocaine-induced changes in NAc astrocytes.

NAc astrocytes are unchanged in female rats following abstinence from LgA cocaine self-administration

Although these changes in NAc astrocytes were observed in male rats following abstinence from LgA cocaine self-administration, the results from experiment 3.2 show that these changes were not observed in female rats. The findings from this experiment suggest a possible protective role of estrogens and other sex hormones against the cocaine-induced changes in NAc astrocytes. However, there was no effect of estrous cycle on the cocaine-induced changes in astrocytes. Similarly to the results discussed in Chapter 2, the same limitations exist in experiment 3.2 as the ones outlined in Chapter 2. These limitations include: the lack of a direct manipulation of the estrous cycle, as well as the timing of estrous cycle monitoring. Furthermore, the low sample size in each estrous cycle phase makes it difficult to assess how the estrous cycle influences the cocaine-induced changes in NAc astrocytes. One possible rationale for this observation is that exposure to cocaine directly impacts cyclicity of the estrous cycle (King et al., 1993; King et al., 1990). Therefore, one option for future experiments is to use ovariectomized rats to directly assess how sex hormones effect the cocaine-induced changes in NAc astrocytes.

As mentioned in Chapter 2, numerous *in vitro* studies have shown that estrogens can protect against the loss of glutamate transporters, including GLT-1 (Liang et al., 2002; Pawlak et al., 2005). Since GLT-1 is primarily expressed on astrocytes, it is reasonable to hypothesize that sex hormones such as estradiol can protect against the cocaine-induced changes in the morphology and synaptic colocalization of NAc astrocytes. Indeed, in cultured astrocytes, a

previous study has shown that estradiol can protect against palmitic acid lipotoxicity by increasing 70 kD heat shock proteins and anti-inflammatory cytokines (Frago et al., 2017). Moreover, the same study showed that the mechanism in which estradiol exerts its protective effects is different in astrocytes cultured from male vs. female rats. In astrocytes cultured from male rats, estradiol decreased pJNK, TNFα and caspase-3 activation (Frago et al., 2017). In astrocytes cultured from female rats, administration of estradiol did not change pJNK or TNFα levels, but did decrease the activity of signaling mechanisms leading to apoptosis (Frago et al., 2017). The results from this study indicate that the mechanism in which estradiol acts as a protective agent is different in male vs. female astrocytes. Whereas estradiol may protect against the cocaine-induced changes in astrocyte morphology and synaptic colocalization in female rats, this may not be the case in astrocytes from male rats. Furthermore, a recent study has shown sex differences in microglia induced phagocytosis of astrocytes during development (VanRyzin et al., 2019). In juvenile female rats, phagocytic microglia engulf significantly less newborn cells in the amygdala, allowing for an increase in astrocyte number and density vs. male rats (VanRyzin et al., 2019). This difference can be one potential rationale for the observed sex difference in the cocaine-induced changes in NAc astrocytes.

In addition to the protective effects of estrogens, another possible explanation for the sex difference in the cocaine-induced changes in astrocyte morphology and synaptic colocalization can be sex differences in response to stress. As mentioned above, astrocytes exhibit a decrease in morphometric properties in response to cocaine exposure, as well as in response to chronic stress and anxiety. Since glucocorticoid receptor stimulation has been linked to the stress-induced changes in astrocyte morphology (Lou et al., 2018; Unemura et al., 2012; Yu et al., 2011), sex differences in response to glucocorticoid receptor activation

may underlie the lack of any cocaine-induced changes in astrocytes from female rats. For example, numerous studies have shown that the physiological and behavioral effects in response to activation of glucocorticoid receptors is more pronounced in males vs. females (Kudielka and Kirschbaum, 2005; Rainville and Hodes, 2019). Moreover, a previous study has shown sex differences in response to deletion of the glucocorticoid receptor in cortico-limbic brain regions (Solomon et al., 2012). Whereas deletion of the receptor results in elevated corticosterone levels and increased depressive-like behaviors in male mice, these effects were not observed in female mice (Solomon et al., 2012). Therefore, since males show an enhanced response to activation of glucocorticoid receptors, stress-induced activation of glucocorticoid receptors during abstinence may lead to the changes in astrocyte morphology and synaptic colocalization in male rats but not female rats.

Another possible rationale for the lack of an effect of LgA cocaine self-administration and prolonged abstinence on astrocytes from the NAc of female rats is a basal sex difference in astrocyte morphology. Within saline self-administering rats, astrocytes from the NAc of female rats exhibited significantly smaller surface area but not volume, compared to astrocytes from the NAc of male rats. Since the peripheral branching properties of astrocytes contribute more directly to surface area as opposed to volume (Hama et al., 2004; Testen et al., 2018), this may indicate that prior to cocaine exposure, astrocytes from female rats may exhibit lower branching complexity compared to astrocytes from male rats. As such, the morphological changes induced by LgA cocaine self-administration and abstinence might not be readily observable. Future studies can examine the branching properties of astrocytes from male vs. female rats to confirm this hypothesis. However, it is notable that there was no basal difference

between NAc astrocytes from male and female rats in volume or synaptic colocalization, while both measures were decreased by cocaine in male rats.

The lack of any changes in astrocytes from the NAc of female rats is in line with results from Chapter 2, which showed no changes in NAc GLT-1 mRNA or protein expression following abstinence from LgA cocaine self-administration. Combined with the data from male rats which showed significant decreases in GLT-1 protein (Fischer-Smith et al., 2012), GLT-1 mRNA (Kim et al., 2018b), and the morphometric properties and synaptic colocalization of astrocytes (experiment 3.1), these results suggest a correlation between GLT-1 expression and the cocaine-induced changes in astrocyte morphology. However, it is unknown if the downregulation in NAc GLT-1 expression is a consequence of the cocaine-induced changes in astrocytes, or if GLT-1 expression is a potential mechanism for the retracted phenotype. This question is examined in detail in Chapter 4.

CHAPTER 4

EXAMINING THE RELATIONSHIP BETWEEN GLT-1 EXPRESSION AND THE COCAINE-INDUCED CHANGES IN NAC ASTROCYTES

Introduction

The goal of Chapter 4 was to selectively and directly examine the relationship between NAc GLT-1 expression and the cocaine-induced changes in NAc astrocytes. Ample evidence from the literature and data reported in preceding chapters indicates a strong correlation between GLT-1 expression and the cocaine-induced changes in the morphometric properties and synaptic colocalization of NAc astrocytes. Most notably in male rats, ShA cocaine self-administration and extinction training results in a downregulation of NAc GLT-1 protein levels (Knackstedt et al., 2010), as well as a decrease in surface area, volume and synaptic colocalization of NAc astrocytes (Scofield et al., 2016b; Testen et al., 2018). Increasing access to cocaine self-administration (from ShA to LgA) and increasing the withdrawal period (from 1 day to 45 days) results in even greater decreases in NAc GLT-1 protein (Fischer-Smith et al., 2012). Likewise, results from Chapter 3 suggest that compared to the morphometric changes in NAc astrocytes after ShA/extinction, the decrease in surface area, volume and synaptic colocalization of NAc astrocytes is exacerbated following LgA/abstinence.

In contrast, neither the changes in NAc GLT-1 expression, nor the structural changes in NAc astrocytes are observed following cocaine exposure in female rats, providing further circumstantial evidence for a relationship between NAc GLT-1 expression and NAc astrocytes.

Results from Chapter 2 showed that NAc GLT-1 protein and mRNA levels are unchanged in female rats following LgA/abstinence. Similarly, results from Chapter 3 showed that the changes in NAc astrocytes following LgA/abstinence are also not observed in female rats. Combined, these results all indicate a correlation between GLT-1 expression and the cocaine-induced alterations in NAc astrocytes. However, it is unclear if the downregulation in GLT-1 expression is a mechanism for the decrease in surface area, volume and synaptic colocalization of NAc astrocytes, or if the downregulation in GLT-1 occurs as a consequence of the cocaine-induced changes in astrocytes, or if these are independent changes which occur in parallel. This question was examined in experiment 4.1.

One way in which GLT-1 expression may be related to the structural plasticity of astrocytes is by acting as a protective agent. GLT-1 is primarily expressed on astrocytes and accounts for approximately 90% of all glutamate uptake (Danbolt, 2001). Loss of GLT-1 has been associated with excitotoxicity and altered glutamatergic signaling (Murphy-Royal et al., 2017; Rothstein et al., 1996; Tzingounis and Wadiche, 2007), evidenced by GLT-1 knockout mice that show lethal spontaneous seizures (Tanaka et al., 1997). Moreover, antisense knockdown of GLT-1 expression exacerbates neuronal death and injury following cerebral ischemia (Rao et al., 2001a; Rao et al., 2001b). In contrast, numerous studies have demonstrated that increasing GLT-1 expression can be neuroprotective (Hu et al., 2015; Pajarillo et al., 2019; Soni et al., 2014). For example, upregulating GLT-1 expression suppresses seizures associated with epilepsy (Sha et al., 2017; Soni et al., 2015). Increased GLT-1 expression is also therapeutic against stroke and cerebral ischemic injuries (Hu et al., 2017; Yang et al., 2012; Zhang et al., 2019a). Furthermore, riluzole is an FDA approved pharmacotherapeutic treatment for amyotrophic lateral sclerosis, and its therapeutic effects can

be attributed at least in part to increased level and activity of GLT-1 (Banasr et al., 2010; Brothers et al., 2013).

Importantly, pharmacological treatment with drugs that increase GLT-1 prevents drug seeking following a period of withdrawal or extinction (Nocito Echevarria et al., 2017; Roberts-Wolfe and Kalivas, 2015). Of the various pharmacotherapeutic compounds that increase GLT-1 levels, one of the most well studied has been the β-lactam antibiotic ceftriaxone. Chronic ceftriaxone treatment upregulates GLT-1 levels, increases glutamate uptake, and importantly, reduces cocaine seeking (Fischer et al., 2013; Knackstedt et al., 2010). Ceftriaxone treatment also attenuates methamphetamine and nicotine place preference, (Alajaji et al., 2013; Philogene-Khalid et al., 2017), as well as alcohol intake (Sari et al., 2016; Stennett et al., 2017). Furthermore, intra-NAc knockdown of GLT-1 expression via an antisense vivo-morpholino prevents ceftriaxone from attenuating cocaine seeking (LaCrosse et al., 2017). This result highlights the importance of restored GLT-1 expression in the ability of ceftriaxone to decrease cocaine seeking. These studies all indicate that upregulating GLT-1 may be a potential pharmacotherapeutic treatment option to treat drug addiction.

Despite ample evidence that suggests ceftriaxone-induced upregulation of GLT-1 expression is therapeutic, the effects of ceftriaxone on the morphology and synaptic colocalization of astrocytes it not as well known. A previous study from our lab has shown that although ceftriaxone treatment did not have an effect on the cocaine-induced decreases in the surface area and volume of NAc astrocytes, it reversed the cocaine-induced decrease in synaptic colocalization (Scofield et al., 2016b). This suggests that ceftriaxone-induced upregulation of GLT-1 may attenuate cocaine seeking by driving astrocyte processes toward synapses, to enhance glutamate uptake and prevent glutamate efflux during reinstatement.

Although this result was promising and indicates a link between GLT-1 expression and the synaptic colocalization of astrocytes, it nonetheless remains unclear whether these are mechanistically-linked adaptations, or correlations. Furthermore, as a broad-spectrum antibiotic, ceftriaxone has numerous effects in addition to upregulating GLT-1 levels (Peterson et al., 2017; Wang et al., 2017). Most notably, ceftriaxone also upregulates expression of the catalytic subunit of the cystine-glutamate exchanger (xCT) (Knackstedt et al., 2010; LaCrosse et al., 2017; Rao et al., 2015). Increasing xCT levels restores basal extracellular glutamate levels, which has also been shown to attenuate cocaine-seeking (Baker et al., 2003; McFarland et al., 2003).

To directly examine the contribution of restored NAc GLT-1 expression in attenuating cocaine seeking, a recent study used an adeno-associated virus (AAV-GFAP-GLT1a) to directly and selectively upregulate GLT-1 levels in the NAc (Logan et al., 2018). Although AAV-GFAP-GLT1a increased expression and activity of GLT-1, it had no effect on cocaine reinstatement (Logan et al., 2018). Since GLT-1 is primarily expressed on astrocytes (Danbolt, 2001), and synaptic colocalization of NAc astrocytes is decreased following ShA cocaine self-administration and extinction training (Scofield et al., 2016b; Testen et al., 2018), one hypothesis for this finding is that although AAV-GFAP-GLT1a induced upregulation of GLT-1, it was not positioned at the synapse to properly reuptake glutamate (Logan et al., 2018). This hypothesis was directly assessed in experiment 4.1 by utilizing two viral vectors: AAV5-GfaABC1D-GLT-1-HA (to overexpress GLT-1) *and* AAV5-GfaABC1D-LckGFP (to label astrocytes and assess synaptic colocalization). This allowed for to direct examination of the association between GLT-1 expression and the cocaine-induced changes in NAc astrocytes. The behavioral effects of GLT-1 overexpression in the NAc on the incubation of cocaine

craving is examined in experiment 4.2. Furthermore, this previous study (Logan et al., 2018), microinjected AAV-GFAP-GLT1a at the end of cocaine self-administration and prior to the start of extinction training. Since NAc GLT-1 protein levels begin to decline following cessation of cocaine self-administration (Fischer-Smith et al., 2012), overexpressing GLT-1 at this time point may not be beneficial in attenuating cocaine seeking. Therefore, to overcome this hurdle, in experiments 4.1 and 4.2, GLT-1 was overexpressed in the NAc prior to the start of self-administration.

Methods

Animals

Male (225-250 g) Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN) and individually housed in temperature and humidity controlled standard plexiglass cages on a reverse light-dark cycle (7 AM off, 7 PM on). All rats were allowed to acclimate to the animal facility for one week, where food and water were available ad libitum. Following the acclimation period, all rats were placed on a food restricted diet of ~ 20 g of chow per day. Food restriction lasted throughout all surgical, post-operative, and food-training procedures. Rats were then returned to an ab libitum diet which lasted throughout the duration of the self-administration phase, and throughout the 45-day abstinence period. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Jugular Vein Catheterization Surgery

Following the one-week acclimation period, all rats were anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg). Prior to the start of surgical procedures, all rats were administered an i.p. injection of the analgesic meloxicam (4 mg/kg). As described in the

previous chapters, a silastic catheter was implanted into the right jugular vein. The catheter ran subcutaneously and exited the back between the shoulder blades where it was capped by sealed Tygon tubing. Catheters were flushed daily with an antibiotic (gentamicin 5 mg/ml, 0.1 mL i.v.) and heparinized saline (100 U/ml, 0.1 mL i.v.) throughout all post-operative and self-administration procedures. Before the start of self-administration procedures, patency of the catheters was examined by administering a sub-threshold dose of propofol (10 mg/mL, 0.05 mL).

LgA Cocaine Self-administration

All food training and self-administration procedures were conducted in standard sound attenuated operant conditioning chambers (Med Associates, St. Albans VT). Prior to the start of cocaine self-administration, to facilitate acquisition to lever pressing, all rats were received one food training session, where lever presses on the active lever resulted in the delivery of one 45 mg food pellet (Bio Serv, Flemington, NJ). Food training sessions lasted a minimum of 6 hours and criteria for food training was set at greater than 100 responses on the active lever. Following food training, all rats underwent LgA cocaine self-administration for 6 hours/day, as previously described in Chapters 2 and 3. A response on the active lever resulted in the delivery of cocaine (0.75 mg/kg/infusion) along with the presentation of audio (70 dB, 2.5 kHz tone) and visual (stimulus light above the active lever) cues for five seconds. A response on the active lever was followed by a 20-sec time out period. Active lever presses during this time resulted in no programmed responses. Responses on the inactive lever at any time during the session were recorded but also resulted in no programmed responses. Following 10 days of LgA cocaine self-administration, all rats underwent experimenter-

induced abstinence in the home cage for 45 days. Throughout the 45-day abstinence period, all rats were handled at least two times per week.

AAV5-GfaABC1D-GLT-1-HA viral vector

AAV5-GfaABC1D-GLT-1-HA was employed in experiments 4.1 and 4.2 to overexpress GLT-1 in the NAc. To generate this viral vector, the his-tagged AAV-GFAP-GLT1a plasmid used in previous experiments (Li et al., 2014; Logan et al., 2018) was obtained and sequenced. Importantly, the ability of this construct to enhance glutamate uptake has been previously verified and validated (Li et al., 2014; Logan et al., 2018). The coding sequence of this plasmid was used to replace the coding sequence of the AAV-GfaABC1D-Lck-GFP plasmid, which was previously used in Chapter 3 and previous experiments to assess cocaine-induced changes in astrocyte morphology (Scofield et al., 2016b; Testen et al., 2019; Testen et al., 2018). Furthermore, the his-tag was removed from the N-terminus of GLT-1 and replaced with a hemagglutinin (HA) tag. The larger peptide sequence of the HA tag and better selectivity of anti-HA antibodies make HA-tagged proteins easier to identify. This generated a new plasmid which overexpressed HA-tagged GLT-1 under control of the astrocyte specific GfaABC1D promoter. All cloning was performed by GenScript (Piscataway, NJ). The new plasmid was then amplified and packaged into an AAV5 serotype by the UNC viral vector core.

Experiment 4.1: Effects of viral GLT-1 overexpression on the cocaine-induced changes in the morphology and synaptic colocalization of NAc astrocytes

Surgical Procedures

To examine the relationship between NAc GLT-1 expression and the cocaine-induced changes in NAc astrocytes, immediately following jugular vein catheter implantation, rats in

experiment 4.1 were microinjected with both AAV5-GfaABC1D-Lck-GFP (to image astrocytes) *and* AAV5-GfaABC1D-GLT-1-HA (to overexpress GLT-1) in the NAc. Equal volumes of AAV5-Lck-GFP (6.1 x 10^{12} virus molecules/mL) and AAV5-GLT-1-HA (9.1 x 10^{12} virus molecules/mL) were gently mixed in a sterile vial. Each rat was then microinjected (bilateral microinjections, 0.1 µL/min, 2 µL per hemisphere) in the NAc (6° angle, AP +1.5, ML +2.6, DV -7.2) with the mixed solution. All rats were given a minimum of 5 days of post-operative care before starting self-administration procedures.

Immunohistochemistry

Following 45 days of abstinence from LgA cocaine self-administration, all rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 1x phosphate buffer (PB), followed by 4% paraformaldehyde (PFA, in 1x PB). Tissues sections (100 µm) from the NAc were collected using a cryostat (Leica Biosystems, Buffalo Grove, IL) and stored in 50% glycerol/PBS until immunostaining.

For immunohistochemistry staining, free-floating sections were first washed (3 x 5 min) in 1x PBS containing 2% Triton X-100 (PBST) (Thermo Fisher, Waltham, MA). Sections were then blocked in 5% normal goat serum (NGS, Sigma Aldrich, St. Louis, MO) in PBST for 1 hour at room temperature. Blocking solution was then replaced with primary antibodies (all at 1:500) in 5% NGS in PBST. The primary antibodies used were mouse anti-PSD-95 (Thermo Fisher, Waltham, MA) and rabbit anti-HA (Cell Signaling Technology, Danvers, MA). Sections were probed for primary antibodies for 72 hours at 4 °C. Slices were flipped halfway through the incubation period to allow maximum penetration of primary antibodies. Following incubation for primary antibodies, secondary antibodies were then added to a solution containing 5% NGS in PBST. Identically to the incubation period for primary

antibodies, slices were the probed for secondary antibodies for 72 hours at 4 °C. The secondary antibodies used were goat anti-mouse Alexa Fluor 594 (Thermo Fisher, Waltham, MA) and goat anti-rabbit Alexa Fluor 647 (Thermo Fisher, Waltham, MA). Following incubation with secondary antibodies, sections were washed 3 x 10 min in PBST, followed by one wash in 1x PBS. Sections were then mounted onto slides and cover slipped with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL).

Astrocyte Image Acquisition and Processing

In experiment 4.1, the Alexa 647 signal was used to determine if Lck-GFP expressing astrocytes also co-expressed GLT-1 (Fig 4.1c). Once Lck-GFP expressing astrocytes were classified as GLT-1 HA+ (Fig 4.2a bottom) or GLT-1 HA- (Fig 4.2a top), all image acquisition and processing of NAc Lck-GFP expressing astrocytes were identical to methods described in Chapter 3. A Zeiss LSM800 confocal-scanning microscope (405/488/561/640 nm diode lasers, 2 GaAsP detectors, 63x oil-immersed objective), along with the following parameters: 1024 x 1024 pixels, bit depth 16-bit, 4x averaging, 1µm z-step, was used for image acquisition. Only single, isolated astrocytes within the NAc were acquired (Fig 4.1e). Astrocytes were not imaged if they were outside of the NAc, bordering and near proximity of other astrocytes, or cut within the z-plane during sectioning.

Following image acquisition, raw images were deconvolved using AutoQuant software (v. X3.0.4, MediaCybernetics) and imported into Imaris software (v 8.4.1, Bitplane, Zurich, Switzerland) for analysis. Using the innate Lck-GFP signal from each astrocyte, each cell was reconstructed in three dimensions and a surface was built around each astrocyte to obtain measurements of surface area and volume. A masked channel was then created to isolate the astrocyte Lck-GFP signal from background, and this masked channel was then used to perform

colocalization analysis between the masked Lck-GFP signal and PSD-95, represented by the Alexa 594 signal. A colocalization channel was then generated to obtain the percentage of masked Lck-GFP signal (defined as the region of interest, ROI) colocalized with PSD-95. For PSD-95 analysis, a 50 µm x 50 µm x 50 µm box was constructed around the isolated astrocyte, and the number of positive PSD-95 puncta above a predetermined threshold was automatically counted. All imaging and analysis were done blind to groups.

Data Analysis

For all astrocyte imaging data, SAS (v. 9.4) software was used to conduct a nested ANOVA. Analysis was performed comparing GLT-1-HA^{+} vs. GLT-1 HA^{-} astrocytes with surface area, volume, synaptic colocalization, or the number of PSD-95 puncta above threshold set as dependent variables.

Experiment 4.2: Effects of viral GLT-1 overexpression on the incubation of cocaine craving

Surgical Procedures

To examine whether overexpression of GLT-1 in the NAc can influence the incubation of cocaine craving, immediately following jugular vein catheter implantation, rats in experiment 4.2 were microinjected with AAV5-GfaABC1D-GLT-1-HA *or* AAV5-GfaABC1D-Lck-GFP in the NAc. Rats microinjected with AAV5-GfaABC1D-LckGFP were used as control rats in the cocaine-seeking test, since no physiological changes in astrocytes were expected to occur with Lck-GFP. Each rat was microinjected (bilateral microinjections, 0.1 µL/min, 1 µL per hemisphere) in the NAc (6° angle, AP +1.5, ML +2.6, DV -7.2). All rats were given a minimum of 5 days of post-operative care before starting self-administration

procedures. Viral expression of GLT-1-HA or Lck-GFP was confirmed in the NAc following the last seeking test (Fig 4.4b and 4.4c).

Tests for Cue-Induced Cocaine-Seeking

To examine the effects of NAc GLT-1 overexpression on the incubation of cocaine craving, a within-subjects design was used to assess time dependent changes in cocaine seeking. Approximately 24 hours following the last LgA cocaine self-administration session, rats microinjected with GfaABC1D-Lck-GFP or GfaABC1D-GLT-1-HA received a 6-hour extinction test to measure cue-induced cocaine seeking. Lever presses on the active lever resulted in the delivery of both audio and visual cues, but not in the delivery of cocaine. Lever presses on the inactive lever resulted in no programmed responses. Following 45 days of abstinence from LgA cocaine self-administration, all rats underwent a second cue-induced seeking test. Time-dependent changes in cocaine seeking were assessed by comparing lever presses across the two seeking tests.

Data Analysis

To measure the effects of GLT-1 overexpression on the incubation of cocaine craving, a mixed ANOVA was performed using SPSS (version 25). A mixed ANOVA ($\alpha = 0.05$) was conducted with group (Lck-GFP (control) vs. GLT-1-HA overexpression) and time (abstinence day 1 vs. abstinence day 45) set as independent variables. The dependent variable was the amount of active lever presses during the seeking test.

Results

Experiment 4.1: Effects of viral GLT-1 overexpression on the cocaine-induced changes in the morphology and synaptic colocalization of NAc astrocytes

To examine the effects of GLT-1 overexpression on the cocaine-induced changes in NAc astrocytes, rats were microinjected with both AAV5-GfaABC1D-GLT-1-HA and AAV5-GfaABC1D-Lck GFP into the NAc at the time of jugular vein catheter surgeries (Fig 4.1a). Behaviorally, all rats underwent LgA cocaine self-administration and demonstrated a clear preference for the active lever (Fig 4.1b). Furthermore, a one-way repeated measures ANOVA showed that all rats exhibited an escalation of cocaine intake across self-administration, with a significantly greater amount of infusions received on days 9 (vs. day 1 $t = 4.59$, $p < 0.001$; vs. day 2 $t = 6.07$, $p < 0.001$; Fig 4.1b) and 10 (vs. day 1 $t = 6.07$, $p < 0.001$; vs. day 2 $t = 7.54$, $p < 0.001$; Fig 4.1b) of LgA cocaine self-administration.

Viral expression of AAV5-GfaABC1D-GLT-1-HA and AAV5-GfaABC1D-Lck-GFP in the NAc is shown is Figure 4.1c (left: Lck-GFP, middle: GLT-1 HA, right: merge of both channels). Importantly, there was significant overlap and colocalization of AAV5-GfaABC1D-GLT-1-HA with AAV5-GfaABC1D-Lck-GFP (Fig 4.1d), indicating expression and spread of AAV5-GfaABC1D-GLT-1-HA. Furthermore, a subset of astrocytes expressed both Lck-GFP and GLT-1 HA (77 cells from 12 rats, Fig 4.1c right panel, co-labeled cells are in white). These Lck-GFP expressing astrocytes were classified as GLT-1-HA-positive (GLT-1-HA$^+$) cells (Fig 4.2a bottom panels). Importantly, a portion of Lck-GFP expressing cells did not co-express GLT-1 (43 cells from 12 rats; Fig 4.1c right, cells that only express Lck-GFP are in green). These Lck-GFP expressing astrocytes were classified as GLT-1-HA-negative (GLT-1-HA$^-$) cells (Fig 4.2a top panels). To examine the effects of viral overexpression of

GLT-1-HA on the cocaine-induced changes in NAc astrocytes, the surface area and volume of GLT-1-HA^{+} vs. GLT-1-HA^{-} astrocytes were assessed (Fig 4.2a). There was no effect of GLT-1 overexpression on the cocaine-induced changes in the morphology of NAc astrocytes. There was no difference between GLT-1-HA^{+} and GLT-1-HA^{-} astrocytes in surface area ($F_{(1, 22)}$ = 0.87, p = 0.36; Fig 4.2b) or volume ($F_{(1, 22)}$ = 0.39, p = 0.54; Fig 4.2c).

To examine if GLT-1 overexpression had an effect on synaptic colocalization of NAc astrocytes, the colocalization of the Lck-GFP signal and the synaptic marker PSD-95 was assessed in GLT-1-HA^{+} vs. GLT-1-HA^{-} astrocytes. There was a statistically significant difference between GLT-1-HA^{+} vs. GLT-1-HA^{-} astrocytes in synaptic colocalization ($F_{(1, 22)}$ = 4.28, p = 0.05; Fig 4.3a and 4.3b). GLT-1-HA^{+} astrocytes exhibited an approximately 14% increase in colocalization with PSD-95 (Fig 4.3b). This difference in synaptic colocalization was not due to any effects of GLT-1 overexpression on PSD-95, as there was no difference between GLT-1-HA^{-} and GLT-1-HA^{+} astrocytes in the number of positive PSD-95 puncta above threshold (Fig 4.3c).

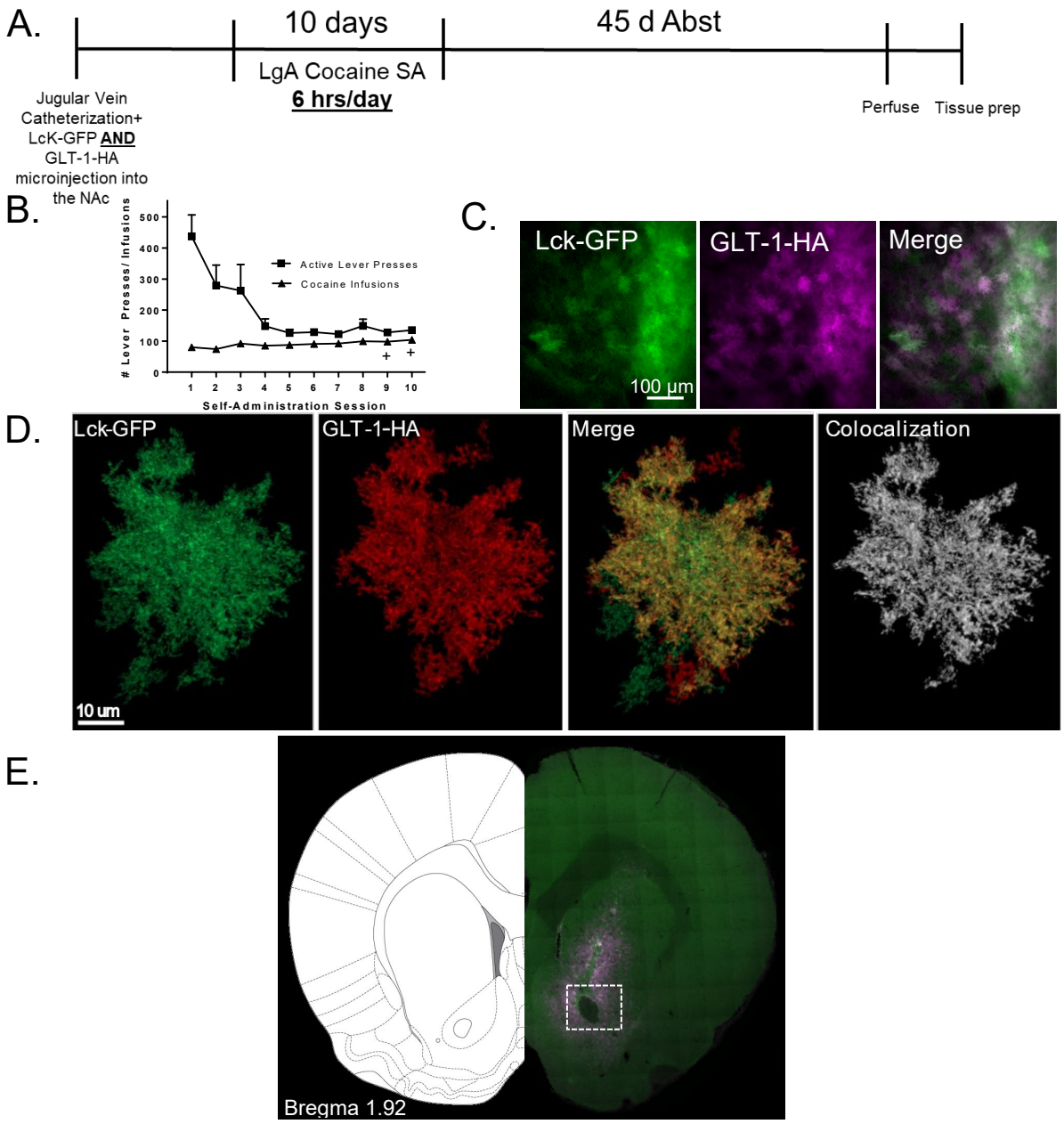

Figure 4.1 Examining the effects of viral overexpression of GLT-1 in the NAc on the cocaine-induced changes in NAc astrocytes. (A) Timeline of experiments. **(B)** Active lever presses and cocaine infusions received during cocaine self-administration. All rats showed a clear preference for the active lever and demonstrated an escalation of cocaine intake (+ $p <$ 0.001; days 9/10 vs. days 1/2 **(C)** 10x confocal image of the NAc containing Lck-GFP astrocytes (left), GLT-1-HA overexpression (middle), and the merge of the two channels (right). **(D)** 63x confocal image demonstrating colocalization of Lck-GFP and GLT-1-HA. **(E)** Image indicating spread of Lck-GFP (green), GLT-1-HA overexpression (purple) and the colocalization of the two (white). Only astrocytes within the white square were used for image analysis.

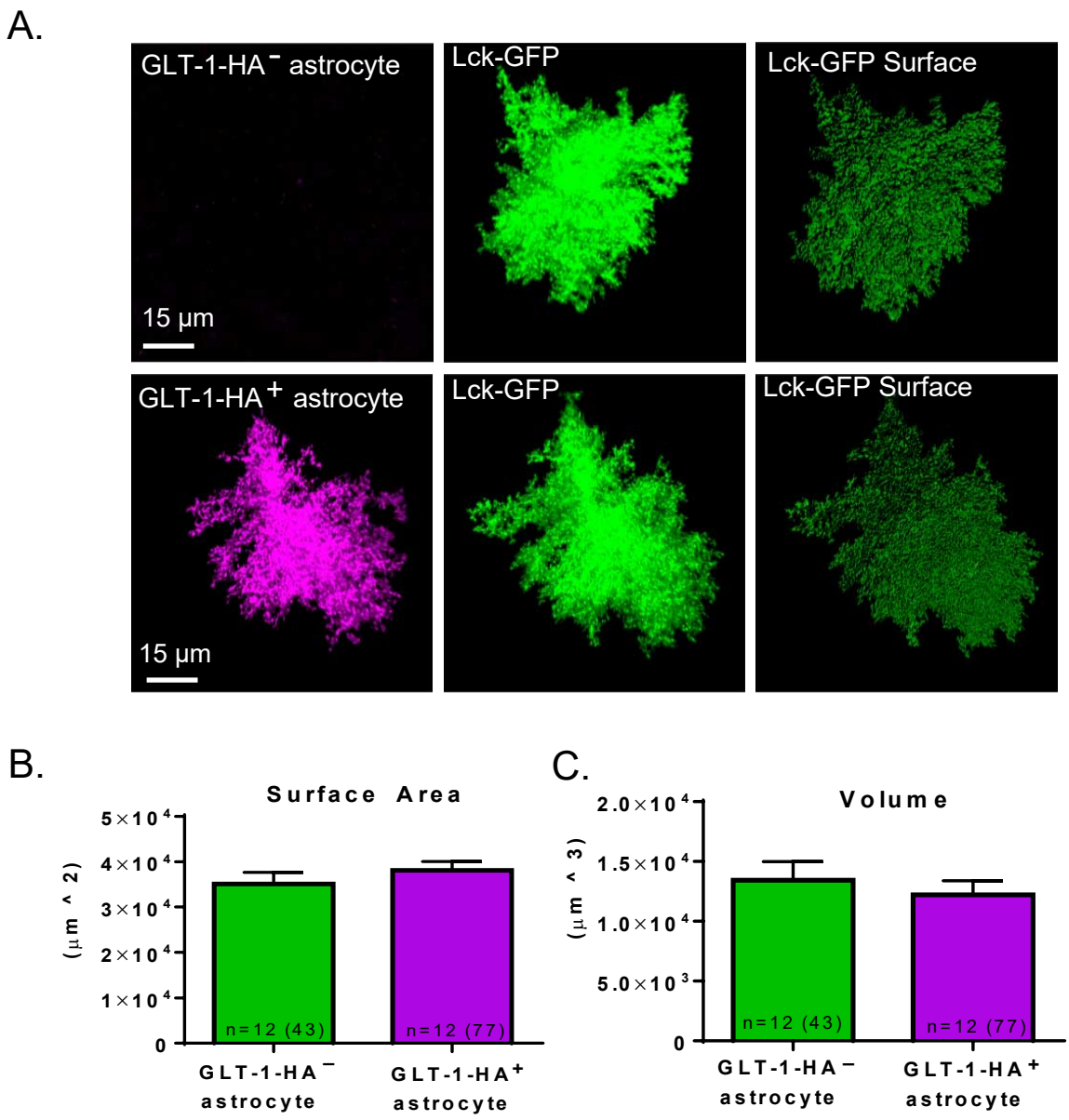

Figure 4.2. Examining the effects of viral overexpression of GLT-1 in the NAc on the cocaine-induced changes in the morphometric properties of NAc astrocytes. (A) 63x confocal image of GLT-1-HA expression (left panels), Lck-GFP expressing astrocyte (middle panels), and surface reconstruction of astrocytes (right panels). A Lck-GFP expressing astrocyte negative for GLT-1 HA overexpression (GLT-1-HA$^-$) is shown in the top panels, and a Lck-GFP expressing astrocyte positive for GLT-1 HA overexpression (GLT-1-HA$^+$) is shown in the bottom panels. There is no difference between GLT-1-HA$^-$ and GLT-1-HA$^+$ astrocytes in **(B)** surface area or **(C)** volume following LgA cocaine self-administration and prolonged abstinence.

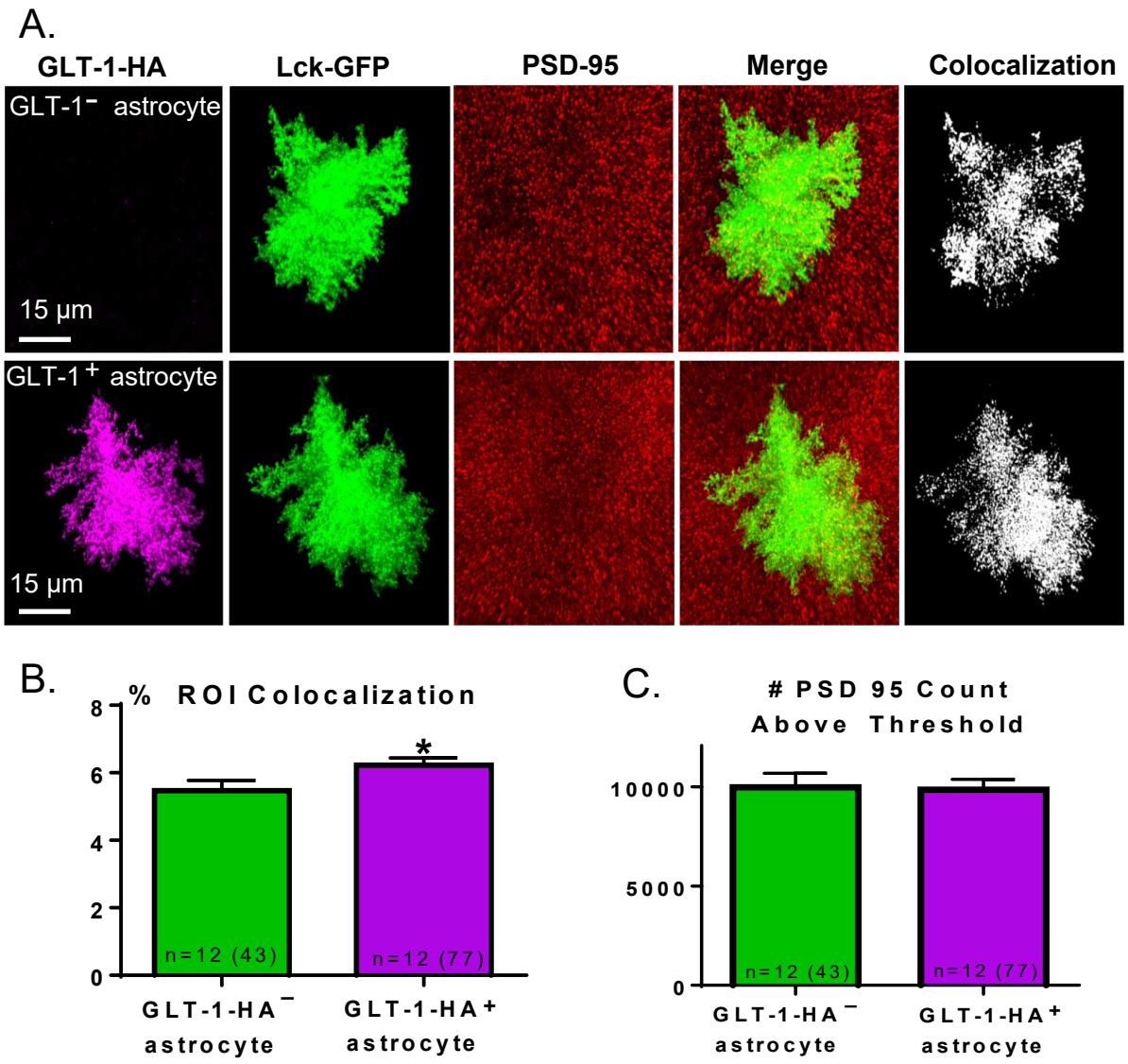

Figure 4.3. Examining the effects of viral overexpression of GLT-1 in the NAc on the cocaine-induced changes in the synaptic colocalization of NAc astrocytes. (A) 63x confocal image of (from left to right): GLT-1-HA, Lck-GFP expressing astrocyte, the synaptic marker PSD-95, merge of Lck-GFP and PSD-95, and colocalization of Lck-GFP and PSD-95 channels. A Lck-GFP expressing astrocyte negative for GLT-1-HA overexpression (GLT-1-HA$^-$) is shown in the top panels, and a Lck-GFP expressing astrocyte positive for GLT-1 HA overexpression (GLT-1-HA$^+$) is shown in the bottom panels. **(B)** GLT-1-HA$^+$ astrocytes show a significant increase in synaptic colocalization vs. GLT-1-HA$^-$ astrocytes. **(C)** The difference in synaptic colocalization is not due to an effect of GLT-1 on the number of positive PSD-95 puncta above threshold.

Experiment 4.2: Effects of viral GLT-1 overexpression on the incubation of cocaine craving

To examine the behavioral effects of GLT-1 overexpression in the NAc, rats were microinjected with AAV5-GfaABC1D-GLT-1-HA in NAc at the time of jugular vein catheter surgeries (Fig 4.4a and 4.4c). For comparison, a different group of rats were microinjected with AAV5-GfaABC1D-Lck-GFP (Fig4.4a and 4.4b). Viral expression in the NAc was confirmed in both groups following the last extinction test (Fig 4.4b and 4.4c). Throughout LgA cocaine self-administration, there were no differences between groups in active lever presses ($F_{(1, 159)} = 1.11$, $p = 0.31$; Fig 4.4d) or the amount of cocaine infusions received ($F_{(1, 159)} = 0.21$, $p = 0.65$; Fig 4.4e). For cocaine infusions, the main effect of time was statistically significant ($F_{(9, 159)} = 11.23$, $p < 0.001$; Fig 4.4e). Both groups showed an increase in the amount of cocaine infusions received on days 9 (Lck-GFP: vs. day 1: $t = 4.16$, $p < 0.001$, vs. day 2: $t = 3.46$, $p < 0.001$; GLT-1-HA: vs. day 1: $t = 4.33$, $p < 0.001$, vs. day 2: $t = 4.18$, $p < 0.001$) and 10 (Lck GFP: vs. day 1: $t = 4.58$, $p < 0.001$, vs. day 2: $t = 3.89$, $p < 0.001$; GLT-1-HA: vs. day 1: $t = 4.91$, $p < 0.001$, vs. day 2: $t = 4.76$, $p < 0.001$) of LgA cocaine self-administration.

To examine if virally-induced overexpression of GLT-1 can influence the incubation of cocaine craving, a within-subjects design was used. All rats were administered a cocaine-seeking test following 1 day of abstinence, and again after 45 days of abstinence to measure time-dependent changes in cocaine-seeking. The incubation of cocaine craving is defined as a significant increase in seeking following 45 days of abstinence (Li et al., 2015; Loweth et al., 2014; Lu et al., 2004). When examining active lever presses during the first hour of the seeking test, there was no significant difference between groups ($F_{(1, 31)} = 0.036$, $p = 0.85$; Fig 4.5a). However, the main effect of time was statistically significant ($F_{(1, 31)} = 24.01$, $p < 0.001$; Fig

4.5a). Pairwise comparisons reveal that both groups exhibited an increase in cocaine seeking following 45 days of abstinence vs. 1 day of abstinence (Lck-GFP: $t = 3.75$, $p < 0.05$; GLT-1-HA: $t = 3.18$, $p < 0.05$; Fig 4.5a). Results were identical when examining cocaine seeking across the entirety of the 6-hour extinction test. There was no difference in seeking between groups after 1 or 45 days of abstinence. However, both groups showed a time-dependent increase in cocaine-seeking after 45 days of abstinence from LgA cocaine self-administration (Lck-GFP: $t = 4.01$, $p < 0.05$; GLT-1-HA: $t = 3.30.$, $p < 0.05$; Fig 4.5b).

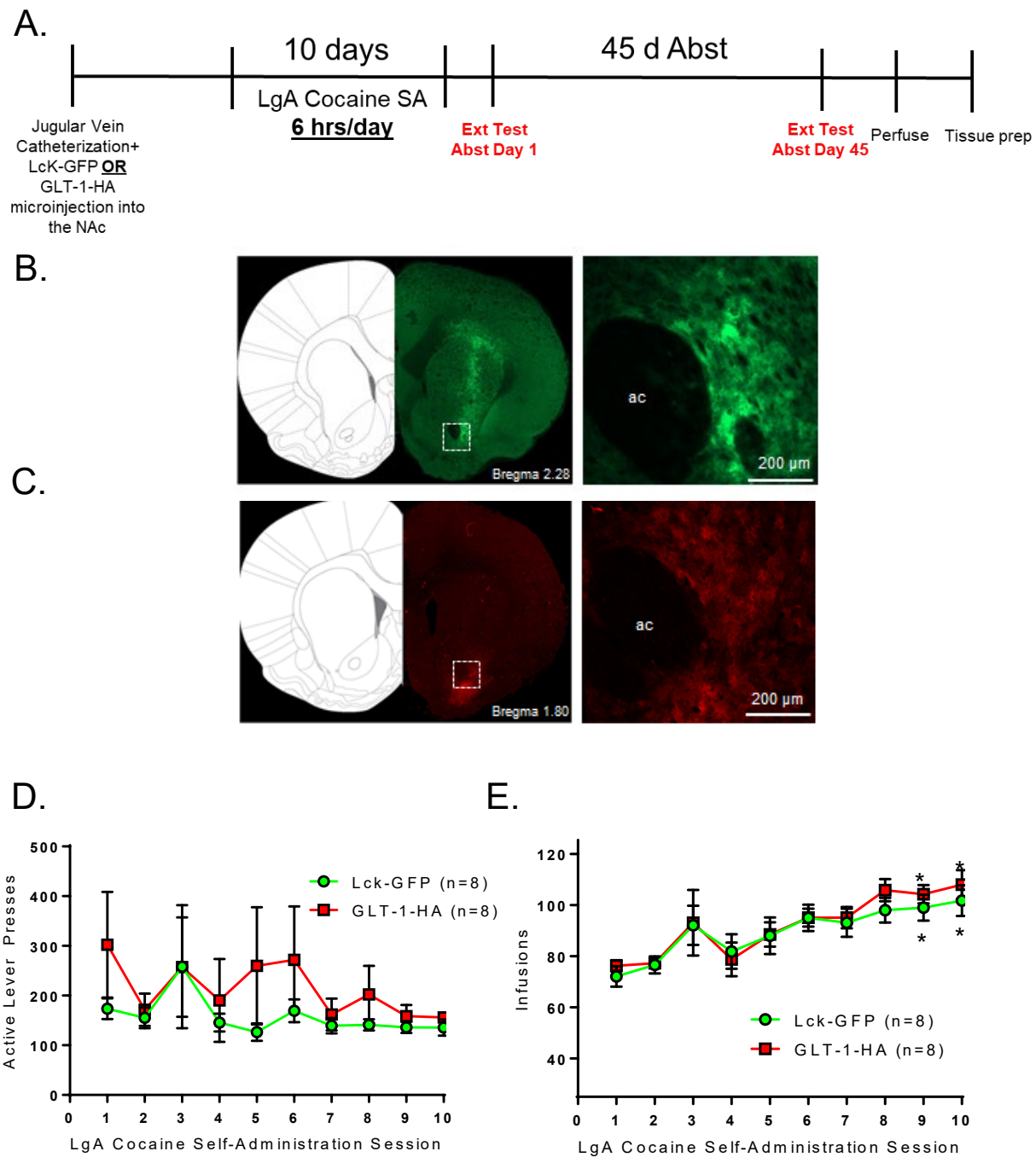

Figure 4.4 Behavioral effects of GLT-1 overexpression in the NAc. (A) Timeline of experiment. **(B)** Confirmation of AAV5-GfaABC1D-Lck-GFP expression in the NAc. **(C)** Confirmation of AAV5-GfaABC1D-GLT-1-HA expression in the NAc. **(D)** Active lever presses and **(E)** cocaine infusions received, in rats that were microinjected with Lck-GFP (green) vs. GLT-1-HA (red). Both groups demonstrate a preference for the active lever and an escalation of cocaine intake (* p <0.001, days 9/10 vs. days 1/2).

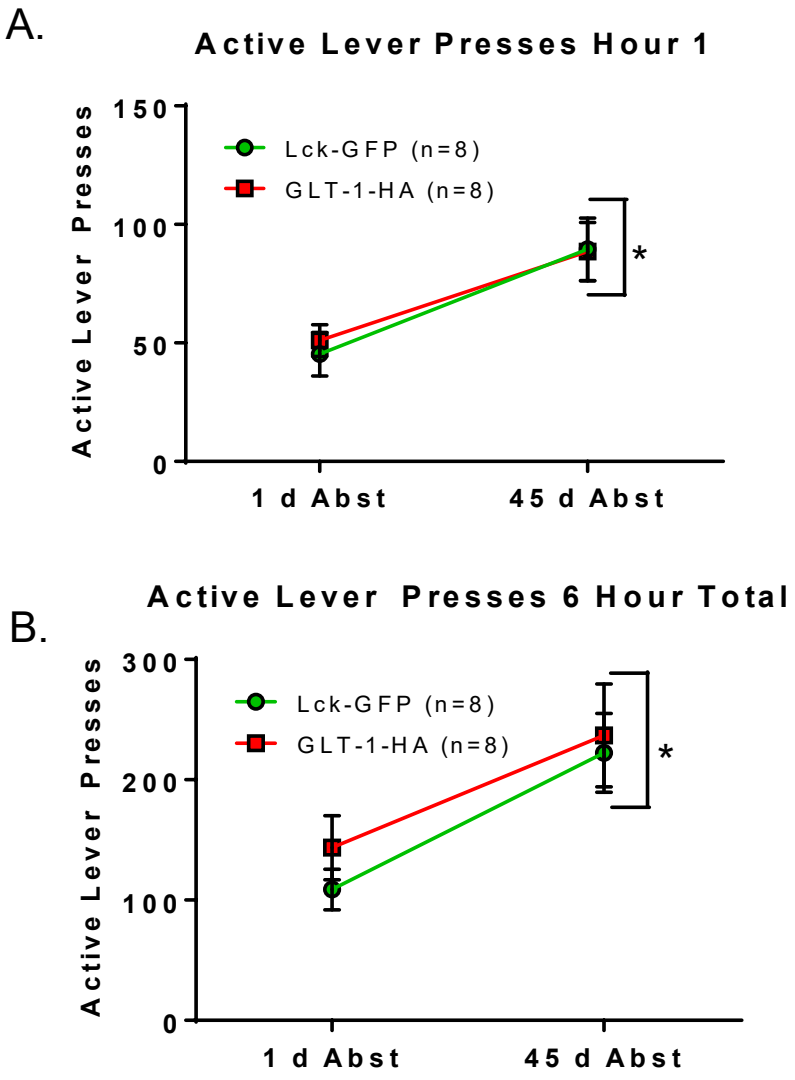

Figure 4.5 GLT-1 overexpression in the NAc has no effect on the incubation of cocaine craving. Following 1 day or 45 days of abstinence from LgA cocaine self-administration, there is no difference between groups in cocaine seeking during **(A)** the first hour or **(B)** the entire 6-hour extinction test, However, both groups exhibited a significant increase in cocaine-seeking following 45 days of abstinence (i.e. an incubation of cocaine craving; * $p < 0.05$ vs. day 1 of abstinence).

Discussion

The results from experiment 4.1 indicate that viral overexpression of GLT-1 in the NAc does not prevent the decrease in surface area and volume of NAc astrocytes following LgA cocaine self-administration and prolonged abstinence. However, in comparison to astrocytes that were negative for GLT-1 overexpression, astrocytes that were positive for GLT-1 overexpression demonstrated a small but significant increase in synaptic colocalization with the synaptic marker PSD-95 (Fig 4.3b). The results from experiment 4.2 indicate that AAV induced viral overexpression of GLT-1 in the NAc does not prevent the incubation of cocaine craving.

Firstly, the results from experiment 4.1 demonstrate the feasibility of utilizing two viral vectors concurrently. In experiment 4.1, AAV-GfaABC1D-Lck-GFP was used to label NAc astrocytes, and AAV-GfaABC1D-GLT-1-HA was used to virally overexpress GLT-1. Although a sizeable portion of Lck-GFP expressing astrocytes co-expressed GLT-1-HA, a subset of Lck-GFP expressing astrocytes did not. This allowed for a direct comparison of the morphometric properties and synaptic colocalization of GLT-1-HA^{+} and GLT-1-HA^{-} astrocytes within the same animal, eliminating potential confounds introduced by using a control virus. Future studies can implement the same techniques and utilize AAV-GfaABC1D-Lck-GFP in conjunction with other viral constructs to examine different aspects of astrocyte structure and function. For example, one possible experiment could concurrently microinject Lck-GFP and astrocyte-specific DREADDs, to examine how manipulating astrocyte function affects astrocyte morphology.

GLT-1 overexpression in the NAc does not influence morphology but increases synaptic colocalization of NAc astrocytes

The results from experiment 4.1 are in line with a previous experiment using ceftriaxone which showed that although chronic ceftriaxone treatment did not prevent the decrease in astrocyte morphology following ShA cocaine self-administration and extinction training, it reversed the decrease in synaptic colocalization (Scofield et al., 2016b). Similarly to these findings, the results from experiment 4.1 illustrate that viral overexpression of GLT-1 in the NAc did not increase the surface area and volume of astrocytes in cocaine-abstinent rats. However, GLT-1 overexpression in the NAc induced a modest but significant increase synaptic colocalization following abstinence from LgA cocaine self-administration. This indicates that the downregulation of NAc GLT-1 expression is likely not a mechanism for the atrophy of astrocytes following withdrawal from cocaine self-administration. Instead, these results show that the downregulation of GLT-1 expression in the NAc may occur either as a consequence of the morphometric changes in astrocytes, or an unrelated parallel effect.

These results further demonstrate that the cocaine-induced changes in the morphometric properties of astrocytes are likely independent from the decrease in the synaptic colocalization. Previous studies have shown that GLT-1 is not expressed at the cell body and is primarily expressed on peripheral astrocytic processes (Melone et al., 2018; Schreiner et al., 2014; Yang et al., 2009). Upregulating NAc GLT-1 expression may then increase synaptic colocalization of astrocytes independent of any changes in astrocyte morphology, by positioning astrocyte processes containing GLT-1 at the synapse, allowing for effective glutamate uptake. Likewise, connexin 30 (Cx30) is a gap junction protein known to play an active role in the adhesion and migration of astrocytes (Ghezali et al., 2018; Pannasch et al., 2014). In the hippocampus of mice, Cx30 induced changes in astrocyte morphology via the

insertion of peripheral astrocytic processes into the synaptic cleft (Pannasch et al., 2014). The positioning of astrocytic processes at the synapse was also associated with an increase in GLT-1 mediated excitatory synaptic transmission (Pannasch et al., 2014). These results show a relationship between GLT-1 expression and the migration of astrocytic processes towards the synapse. Therefore, it remains plausible that viral-induced upregulation of GLT-1 in the NAc increases synaptic colocalization of astrocytes by positioning GLT-1 containing peripheral astrocytic processes at the synapse in order to perform efficient glutamate uptake. Potential mechanisms in which upregulation of GLT-1 can accomplish enhanced synaptic colocalization of astrocytes is via astrocytic neuroligins and astrocyte derived adhesion molecules. Astrocytic neuroligins play a vital role in the morphogenesis and synaptogenesis of astrocytes (Stogsdill et al., 2017). Likewise, astrocyte derived adhesion molecules have also been shown to play an active role in the migration of astrocytes towards neuronal synapses (Cardenas et al., 2014; Liddelow and Hoyer, 2016). However, it is currently known how GLT-1 is related to the expression of astrocytic neuroligins and adhesion molecules, and remains an interesting area of research for future research.

GLT-1 overexpression in the NAc is not sufficient to attenuate cocaine seeking

Although GLT-1 overexpression increased synaptic colocalization of NAc astrocytes, this increase was not sufficient to attenuate cocaine seeking. Results from experiment 4.2 indicate that cocaine seeking was unchanged between rats microinjected with AAV-GfaABC1D-GLT-1-HA and rats microinjected with AAV-GfaABC1D-Lck-GFP. Moreover, both groups demonstrated an incubation of cocaine craving, as cocaine seeking was potentiated in both groups following 45 days of abstinence. The results from experiment 4.2 are in line with a previous finding showing that GLT-1 overexpression does not decrease cocaine seeking

following ShA cocaine self-administration and extinction training (Logan et al., 2018). Despite the fact that previous studies indicate that GLT-1 upregulation is an important mechanistic component of pharmacological inhibition of cocaine reinstatement (discussed below), these results suggest that selectively upregulating GLT-1 alone is not sufficient to reduce cocaine seeking.

The results from experiment 4.2 were surprising considering the ample evidence from previous studies which suggest that pharmacotherapeutic drugs that upregulate GLT-1 can attenuate drug seeking (Knackstedt et al., 2010; Reissner et al., 2014; Reissner et al., 2015; Sari et al., 2016; Sepulveda-Orengo et al., 2018). Moreover, antisense suppression of GLT-1 expression has shown that restored NAc GLT-1 expression is necessary for pharmacological drugs such as ceftriaxone, N-acetylcysteine and propentofylline to decrease drug seeking (LaCrosse et al., 2017; Namba et al., 2019; Reissner et al., 2014; Reissner et al., 2015). However, these pharmacological drugs have a number of effects in addition to upregulating GLT-1 expression. For example, ceftriaxone is a broad-spectrum antibiotic that also increases xCT expression (Knackstedt et al., 2010; LaCrosse et al., 2017), N-acetylcysteine augments cystine-glutamate exchange (Nocito Echevarria et al., 2017), and propentofylline is a non-specific glial modulator (Sweitzer and De Leo, 2011). Therefore, these pharmacological drugs likely engage diverse mechanisms which work in concert with increased GLT-1 expression to attenuate drug seeking. Furthermore, other studies have highlighted the importance of metabotropic and ionotropic glutamate receptors on cocaine seeking (Li et al., 2018; Loweth et al., 2014; Lu et al., 2012; Pomierny-Chamiolo et al., 2017; Schmidt et al., 2015). These results suggest that although restored GLT-1 expression is necessary for some pharmacological compounds to attenuate cocaine seeking, GLT-1 restoration itself is not sufficient to produce

a behavioral response. Other components of glutamate homeostasis, including xCT and glutamate receptors can also influence cocaine-seeking.

Although NAc GLT-1 overexpression induced a significant increase in synaptic colocalization of NAc astrocytes, the synaptic colocalization of GLT-1-HA^{+} astrocytes did not return to synaptic colocalization levels as seen in saline self-administering rats observed in previous experiments. This suggests that perhaps a certain threshold in synaptic colocalization must be reached before a behavioral response can be observed. These results also suggest that GLT-1 overexpression is not sufficient to restore synaptic colocalization to basal levels. This may partially explain why GLT-1 overexpression by itself is not sufficient to attenuate cocaine seeking. Alternatively, the lack of an effect of GLT-1 overexpression on the cocaine-induced decrease in astrocyte morphology, as well as on the incubation of cocaine craving could be attributable to an inadequate number of cells transduced. However, histological analysis indicates significant spread and overexpression of AAV-GfaABC1D-GLT-1-HA (experiment 4.2; Fig 4.4c), as well as significant spread and overexpression of AAV-GfaABC1D-GLT-1-HA in conjunction with AAV-GfaABC1D-Lck-GFP (experiment 4.1; Fig 4.1). Future studies can aim to reverse the cocaine-induced decrease in synaptic colocalization and incubation of cocaine craving by alternate means. One potential candidate for future experiments is manipulating the gap junction protein Cx30. As mentioned above, Cx30 plays an active role in the adhesion and migration of astrocytes (Pannasch et al., 2014). By increasing expression and/or activity of Cx30 in combination with GLT-1 overexpression, it may then be possible to drive synaptic colocalization towards basal levels, which may in turn attenuate cocaine seeking.

CHAPTER 5

GENERAL DISCUSSION

Summary of findings

The experiments and results presented herein provide insights into the regulation of gene expression and morphological features of NAc astrocytes following prolonged abstinence from cocaine self-administration in both male and female rats. Specifically, I found that although the decrease in the morphometric properties and synaptic colocalization of NAc astrocytes are pronounced in male rats, these adaptations are not observed in female rats. Further, the results also show that NAc GLT-1 expression is unchanged in female rats following prolonged abstinence from LgA cocaine self-administration, despite the fact that the GLT-1 gene is silenced via DNA methylation in male rats (Kim et al., 2018b). Finally, the results from this dissertation also show that while upregulating GLT-1 in male rats leads to a marginal but significant increase in synaptic colocalization of NAc astrocytes, this increase is not sufficient to attenuate cocaine seeking behavior.

Data presented in Chapter 2 reveal that prolonged abstinence from LgA cocaine self-administration does not affect GLT-1 mRNA (Fig 2.2a-d; Table 1) or protein levels (Fig 2.4a-c; Table 1) in the NAc of female rats. The results from experiment 2.1 were in contrast to preliminary findings for this dissertation which showed a significant decrease in GLT-1 mRNA levels in male rats (Kim et al., 2018b). Moreover, the results from experiment 2.2 were also in contrast to a previous study which showed a downregulation in NAc GLT-1 protein expression

in male rats following LgA cocaine self-administration and prolonged abstinence (Fischer-Smith et al., 2012).

Subsequently, Chapter 3 provided further evidence for a lack of an effect of LgA cocaine self-administration and abstinence on astrocytes from the NAc of female rats. Although astrocytes from the NAc of male rats displayed a significant cocaine-dependent decrease in surface area (Fig 3.2d; Table 1), volume (Fig 3.2e; Table 1) and synaptic colocalization (Fig 3.3a-b; Table 1), these effects were not observed in NAc astrocytes from female rats (Fig 3.4d-e, Fig3.6a-b; Table 1). These findings add to the existing literature, and further suggest a correlation between the cocaine-induced downregulation in NAc GLT-1 expression and the cocaine-induced structural adaptations of NAc astrocytes. However, whether these findings are correlational or mechanistically linked could not be addressed by those findings. This question was directly examined in Chapter 4.

Data presented in Chapter 4 suggest that while viral induced overexpression of GLT-1 in the NAc has no effect on the cocaine-induced decrease in the morphometric properties of astrocytes (Fig 4.2a-c), NAc GLT-1 overexpression modestly but significantly increased synaptic colocalization (Fig 4.3a-b). However, this increase in synaptic colocalization was independent of any behavioral effects, as GLT-1 overexpression did not prevent the incubation of cocaine craving (Fig 4.4). This suggests that the downregulation of the structural features of astrocytes is likely not a consequence of reduced glutamate uptake. Furthermore, the data from Chapter 4 also indicate that the morphometric changes in NAc astrocytes from male rats following LgA cocaine self-administration and prolonged abstinence are independent from the changes in synaptic colocalization.

A summary of the findings in male rats is presented in Figure 5.1. Under drug naïve conditions, GLT-1 is positioned on the peripheral processes of astrocytes to perform efficient glutamate uptake (Fig 5.1a). Following prolonged abstinence from LgA cocaine self-administration, NAc GLT-1 expression is downregulated, and NAc astrocytes exhibit a significant decrease in surface area, volume and synaptic colocalization (Fig 5.1b). GLT-1 overexpression in the NAc has no effect on the morphology but partially restores synaptic colocalization of NAc astrocytes following LgA/abstinence (Fig 5.1c). However, this increase alone is not sufficient attenuate cocaine seeking. This raises the question of whether enhanced stimulation of NAc astrocytes is needed to augment synaptic colocalization and/or attenuate the incubation of cocaine craving (Fig 5.1d).

Existing evidence indicates that stimulation of NAc astrocytes can oppose cocaine seeking behavior. For example, stimulation of astrocyte-specific Gq-coupled DREADDs leads to decreased reinstatement after ShA cocaine self-administration and extinction training (Scofield et al., 2015), as well as alcohol seeking (Bull et al., 2014). However, it is unknown if these findings extend to the incubation of cocaine craving. Furthermore, it is unknown if DREADD stimulation of NAc astrocytes influences the cocaine-induced decrease in the morphometric properties and synaptic colocalization of astrocytes. A hypothesis for how chemogenetic stimulation of NAc astrocytes may increase synaptic colocalization via actin dynamics is shown in Figure 5.1d.

The findings from this dissertation add to the results from previous studies (both summarized in Table 1), and indicate a common theme across two commonly utilized rodent models of drug addiction. ShA cocaine self-administration and extinction training results in a downregulation in NAc GLT-1 protein levels (Fischer-Smith et al., 2012; Knackstedt et al.,

2010), as well as a decrease in the surface area, volume and synaptic colocalization of NAc astrocytes (Scofield et al., 2016b; Testen et al., 2018). LgA cocaine self-administration and prolonged abstinence, associated with the incubation of cocaine craving results in an even further decrease in NAc GLT-1 protein levels (Fischer-Smith et al., 2012), a downregulation in GLT-1 mRNA (Kim et al., 2018b), and substantial decreases in the morphometric properties and synaptic colocalization of NAc astrocytes (Chapter 3). Although the two models have varied features (i.e. 2 vs. 6 hours of cocaine self-administration; extinction vs. abstinence), they share a common theme of consequences of cocaine self-administration on GLT-1 expression and structural features of astrocytes. Furthermore, the effects of cocaine on these measures are generally more pronounced following LgA/abstinence. Despite some evidence of a downregulation in NAc GLT-1 protein in female rats following ShA/extinction (Bechard et al., 2018), these effects are somewhat specific to male rats, as female rats show no changes in NAc GLT-1 protein (Chapter 2), NAc GLT-1 mRNA (Chapter 2) or NAc astrocytes (Chapter 3) following LgA/abstinence. The implications of these findings are discussed in further detail below.

Changes in the NAc	ShA cocaine self-administration and extinction training		LgA cocaine self-administration and prolonged abstinence	
	♂	♀	♂	♀
GLT-1 expression	↓ (Knackstedt et al, 2010)	↓ (Bechard et al, 2018)	↓↓↓ (Fischer-Smith et al, 2012; Kim et al, 2018)	**No change** (Chapter 2)
Surface area and volume of astrocytes	↓ (Scofield et al, 2016; Testen et al, 2018)	**TBD**	↓↓↓ (Chapter 3)	**No change** (Chapter 3)
Synaptic colocalization of astrocytes	↓ (Scofield et al, 2016; Testen et al, 2018)	**TBD**	↓↓↓ (Chapter 3)	**No change** (Chapter 3)

Table 1. Summary of the changes in NAc GLT-1 expression (GLT-1 protein after ShA/extinction; GLT-1 protein and mRNA after LgA/abstinence), the morphometric properties of NAc astrocytes, and the synaptic colocalization of NAc astrocytes in male and female rats following two widely employed rodent models of addiction, ShA/extinction model (left), and the LgA/abstinence incubation model (right).

Sex differences in the structural adaptations of NAc astrocytes following prolonged abstinence from LgA cocaine self-administration

Ample evidence from previous studies has demonstrated sex differences in cocaine self-administration (Becker, 2016; Kippin et al., 2005; Lacy et al., 2016; Lynch and Carroll, 1999). Female rats generally acquire cocaine self-administration more readily, exhibit higher lever pressing during maintenance of cocaine self-administration and show higher motivation to lever press for cocaine (Hu et al., 2004; Jackson et al., 2006; Lynch, 2008; Lynch and Carroll, 1999; Roth and Carroll, 2004; Swalve et al., 2016; Zhao and Becker, 2010). However, other studies have reported the opposite effect (Caine et al., 2004), or no sex difference (Jordan and Andersen, 2018), effects possibly mediated by cocaine dose, as well as reinforcement schedule.

In addition, numerous studies have established effects of the estrous cycle on cocaine self-administration. For example, female rats in estrus show a higher breakpoint during a progressive ratio test, as well as greater cocaine-seeking during reinstatement (Kippin et al., 2005; Lacy et al., 2016). Furthermore, similarly to male rats, female rats also display an incubation of cocaine craving (Nicolas et al., 2019; Zlebnik and Carroll, 2015), which is more pronounced during the estrus stage (Nicolas et al., 2019). Despite these well-established sex-dependent observations in cocaine self-administration, very few studies to date have examined glutamatergic signaling and/or astrocyte changes in female rats following abstinence from cocaine self-administration.

The results from Chapter 3 are in line with the previous studies mentioned above and show that female rats received a higher number of cocaine infusions than male rats during self-administration (Fig 3.8). Despite this, the results from Chapters 2 and 3 suggest that female

rats may be resistant to the cocaine-induced downregulation in NAc GLT-1 expression (Fig 2.2 and 2.4), as well as the cocaine-induced decrease in surface area, volume and synaptic colocalization of NAc astrocytes (Fig 3.5 and 3.7). These results were surprising considering that male rats exhibit a substantial downregulation in NAc GLT-1 protein (Fischer-Smith et al., 2012), decrease in NAc GLT-1 mRNA (Kim et al., 2018b), and significant decreases in surface area, volume and synaptic colocalization of NAc astrocytes (Chapter 3) following abstinence from LgA cocaine self-administration (Table 1).

The lack of any observable effects on astrocytes in female rats is in line with previous studies suggesting that astrocytes from female rodents are generally protected against various forms of injury (Frago et al., 2017; Liang et al., 2002; Morizawa et al., 2012; Pawlak et al., 2005). For example, estrogen enhances astrocytic glutamate uptake (Liang et al., 2002; Pawlak et al., 2005), increases expression of Hsp70 in astrocytes (Frago et al., 2017) and decreases apoptosis in astrocytes (Frago et al., 2017). However, the exact mechanism for this phenomenon remains unclear. Previous studies have shown that the presence of estrogen can impact various signaling mechanisms leading to an increase in astrocyte number and density (Frago et al., 2017; VanRyzin et al., 2019). Similarly, estrogen increases GFAP density and expression in multiple different brain regions (Arias et al., 2009; Siani et al., 2017; Zsarnovszky et al., 2002). Interestingly, the α and β forms of the estrogen receptor are expressed on astrocytes (Azcoitia et al., 2001; Dhandapani and Brann, 2007). The activation of these estrogen receptors plays a critical role in the neuroprotective properties of estrogen (Azcoitia et al., 2001; Dhandapani and Brann, 2003; Dhandapani and Brann, 2007). For example, activation of the β receptor is neuroprotective against ischemia (Ma et al., 2016). Likewise, activation of the α receptor is protective against traumatic brain injuries (Lim et al.,

2018; Stary et al., 2017). One possible mechanism by which activation of astrocytic estrogen receptors can be neuroprotective is by initiating feedback signaling mechanisms within astrocytes. This may induce various signaling pathways that protect against downregulation of astroglial proteins such as GLT-1, as well as against the cocaine-induced decrease in the morphometric properties and synaptic colocalization. This is an interesting avenue for future research and remains to be studied.

Although the literature examining cocaine-induced changes in glutamate homeostasis in female rats is sparse, one recent study examined the effects of ceftriaxone on NAc GLT-1 protein expression and reinstatement following ShA cocaine self-administration and extinction training in female rats (Bechard et al., 2018). This study showed that in comparison to a control group, regardless of estrous cycle stage, female rats exhibited a decrease in NAc GLT-1 protein levels (Bechard et al., 2018). Moreover, ceftriaxone treatment increased NAc GLT-1 protein levels in female rats that had previously self-administered cocaine (Bechard et al., 2018). However, the lack of a saline self-administering control group, as well as the small sample size used in this study (n = 8 female rats in estrus, n = 7 non-estrus female rats) make these findings difficult to interpret. Although the results from this study showed a decrease in NAc GLT-1 expression following ShA/extinction, the results from Chapter 2 suggest that this is not an adaptation that occurs in female rats following LgA/abstinence. Preliminary data for this dissertation indicates that in male rats, GLT-1 expression is differentially regulated based on cocaine self-administration paradigm (Kim et al., 2018b). Therefore, it remains plausible that in female rats, regulation of GLT-1 expression may also be dependent on self-administration paradigm.

The study by Bechard et al. also showed that similarly to male rats, ceftriaxone was effective in attenuating reinstatement in female rats (Bechard et al., 2018). However, if the estrous cycle was accounted for, ceftriaxone had no effect in female rats that were tested during the estrus stage (Bechard et al., 2018). Since ceftriaxone upregulated GLT-1 in female rats regardless of estrous cycle stage, this would suggest that in female rats tested during the estrus stage, other mechanisms may drive cocaine-seeking. Indeed, this study found that during the estrus stage, ceftriaxone increased surface expression of the GluA1 AMPA receptor (Bechard et al., 2018). The results from this study suggest that in addition to GLT-1 expression, other mechanisms may impact cocaine-seeking in female rats. For example, it is possible that other neurotransmitter systems beyond glutamate may differentially drive cocaine-seeking in female rats. Previous studies have shown estrous cycle dependent changes in dopamine (Calipari et al., 2017), noradrenaline (Kohtz and Aston-Jones, 2017), and serotonin (Kohtz and Aston-Jones, 2017), all of which have been found to influence cocaine-seeking. However, how these systems interact with the estrous cycle to produce neurobiological adaptations that drive cocaine-seeking in female rats remains to be studied.

On the relationship between GLT-1 expression and the changes in NAc astrocytes following abstinence from LgA cocaine self-administration

The results from Chapters 2 and 3 add to the existing literature and further suggest a correlation between the downregulation in NAc GLT-1 expression and the changes in NAc astrocytes following withdrawal from cocaine self-administration (Table 1). For example, following ShA cocaine self-administration and extinction training, numerous experiments have shown a decrease in NAc GLT-1 protein expression (Knackstedt et al., 2010; Sepulveda-Orengo et al., 2018; Sondheimer and Knackstedt, 2011), as well as a decrease in the surface

area, volume, and synaptic colocalization of NAc astrocytes (Scofield et al., 2016b; Testen et al., 2018). Furthermore, these effects on NAc GLT-1 expression are more prominent following prolonged abstinence from LgA cocaine self-administration (Table 1). For example, 45 days of abstinence from LgA cocaine self-administration leads to a greater downregulation in NAc GLT-1 expression (Fischer-Smith et al., 2012; Kim et al., 2018b). The results from Chapter 3 add to these findings and suggest that the decrease in the morphometric properties and synaptic colocalization of NAc astrocytes is greater following LgA cocaine self-administration and prolonged abstinence (Fig 3.2 and 3.3). Finally, the lack of an effect on NAc GLT-1 expression, as well as the absence of any changes on NAc astrocytes in female rats further suggests an association between GLT-1 expression and the morphology of astrocytes.

These results raise the question of whether the downregulation in GLT-1 expression is mechanistically linked to changes in NAc astrocytes, or if the decrease in GLT-1 expression occurs as a consequence of the cocaine-induced changes in the morphometric properties and synaptic colocalization of NAc astrocytes. The results from Chapter 4 indicate that similarly to a previous experiment using chronic ceftriaxone treatment (Scofield et al., 2016b), AAV-induced GLT-1 overexpression in the NAc had no effect on the cocaine-induced changes in the morphometric properties of NAc astrocytes (Fig 4.2b-c). However, results from experiment 4.1 also show that GLT-1 overexpression in the NAc significantly increased synaptic colocalization with PSD-95 (Fig 4.3a-b; Fig 5.1c). Firstly, these results suggest that the changes in astrocyte morphology are independent of the decrease in synaptic colocalization with PSD-95. Secondly, this result suggests that the downregulation in GLT-1 is likely not a mechanism for the changes in NAc astrocytes following abstinence from LgA cocaine self-

administration, suggesting that the relationship between GLT-1 expression and astrocyte morphology may be correlational and not mechanistically linked.

One alternative hypothesis is that the decrease in surface area and volume of NAc astrocytes following prolonged abstinence from LgA cocaine self-administration leads to the downregulation of astroglial proteins such as GLT-1. In support of this possibility, ShA cocaine self-administration and extinction training has also been shown to reduce other astroglial proteins, including xCT (Knackstedt et al., 2010) and GFAP (Scofield et al., 2016b). Furthermore, current studies in our lab are investigating the changes in the morphology of NAc astrocytes in greater detail. Filament analysis of the branching properties of NAc astrocytes suggest that abstinence from LgA cocaine self-administration leads to a pruning, rather than a shrinkage of NAc astrocytes. The loss of the branching properties of NAc astrocytes may then account for the decrease in the morphometric properties of NAc astrocytes following abstinence from LgA cocaine self-administration (Fig 5.1b; Testen et al, unpublished findings). Since GLT-1 is primarily expressed on peripheral astrocytic processes (Melone et al., 2018; Schreiner et al., 2014), the pruning of these branches is one possibility resulting in the decrease in NAc GLT-1 expression following abstinence from LgA cocaine self-administration (Fig 5.1b).

It is important to note that a decrease in the morphometric features of astrocytes has only been reported after contingent cocaine administration. Numerous studies have reported an increase in GFAP expression following non-contingent cocaine exposure. For example, acute and chronic i.p. injections of cocaine lead to increased GFAP expression in the hippocampus (Blanco-Calvo et al., 2014; Fattore et al., 2002; Zhu et al., 2016). Other studies have reported that withdrawal from non-contingent cocaine administration also leads to

increased GFAP expression in numerous brain regions including the NAc, hippocampus and prefrontal cortex (Bowers and Kalivas, 2003; Fattore et al., 2002; Zhu et al., 2016). The findings from these studies suggest that the atrophy of astrocytes is a phenotype unique to withdrawal from contingent drug administration.

In other neurological disorders, an inverse correlation is often found between GLT-1 levels and changes in GFAP expression. For example, following epilepsy or ischemia, a decrease in GLT-1 levels is often found in conjunction with an increase in GFAP expression (Fukamachi et al., 2001; Nonose et al., 2018; Samuelsson et al., 2000; Schreiner et al., 2013; van Landeghem et al., 2001). This indicates that in neurological disorders such as epilepsy and ischemia, excitotoxicity induced by a loss of GLT-1 is associated with astrogliosis. Similarly, although pharmacological compounds can normalize either GLT-1 or GFAP levels, expression of the other often remains unchanged. For example, following traumatic brain injury, ceftriaxone increases GLT-1 expression, but had no effect on GFAP and hyperreactivity of astrocytes (Goodrich et al., 2013). Likewise, in a rodent model of Parkinson's disease, riluzole reduces GFAP expression, but had no effect on GLT-1 levels (Carbone et al., 2012). The findings from the above-mentioned studies indicate that the correlation between the downregulation in GLT-1 expression and the atrophy of astrocytes is a phenomenon unique to withdrawal from contingent cocaine administration.

The previous experiments that have examined the relationship between GLT-1 and GFAP expression have numerous caveats. First, as mentioned previously, not all astrocytes express GFAP (Kettenmann and Verkhratsky, 2011; Kimelberg, 2004), and further, GFAP expression is highly brain region dependent (Sofroniew, 2009; Sofroniew and Vinters, 2010). Thus, to better understand the relationship between GLT-1 expression and the changes in the

morphometric features of astrocytes, future studies can examine the correlation between GLT-1 expression and other astrocyte markers, such as the calcium binding protein S100β or aldehyde dehydrogenase 1 L1 (ALDH1L1). Second, GFAP only constitutes roughly 15% of the total volume of an astrocyte (Benediktsson et al., 2005; Rajkowska and Stockmeier, 2013). Since GFAP is a cytoskeletal marker for astrocytes (Shigetomi et al., 2013; Shigetomi et al., 2010), changes in GFAP expression may not be indicative of any morphological changes that occur at the astrocytic end feet. This is especially concerning since GLT-1 expression is primarily expressed at the peripheral processes of astrocytes (Melone et al., 2018; Schreiner et al., 2014; Yang et al., 2009). Consequently, changes in GFAP expression may not be a good indicator of changes in GLT-1 levels, and vice versa. Therefore, as outlined in Chapters 3 and 4, utilizing AAV-GfaABC1D-Lck-GFP is a better indicator of the structural and morphometric changes in astrocytes. AAV-GfaABC1D-Lck-GFP expressing astrocytes cover an approximately 10-fold larger area than astrocytes that only express GFAP (Shigetomi et al., 2013). Importantly, this includes Lck-GFP expression at the astrocytic end-feet (Fig 3.1a and 3.1b). As such, changes in astrocyte morphology assessed using Lck-GFP may be a better correlated with the changes in GLT-1 expression and vice versa.

Although the results from this dissertation and previous studies suggest a correlation between GLT-1 expression and cocaine-induced changes in NAc astrocytes (Table 1), results from Chapter 4 suggest that these phenomena may not be mechanistically linked. Therefore, it remains plausible that the downregulation of NAc GLT-1 levels and the decrease in the morphometric properties and synaptic colocalization of NAc astrocytes are guided by different mechanisms. Although the exact basis for the decrease in astrocyte morphology and synaptic colocalization following abstinence from LgA cocaine self-administration has yet to be

determined, preliminary data for this dissertation have implicated epigenetic mechanisms for the observed downregulation of NAc GLT-1 expression following abstinence from LgA cocaine self-administration (Kim et al., 2018b). The results from this preliminary study showed that one mechanism for the observed decrease in NAc GLT-1 protein expression following abstinence from LgA cocaine self-administration is a decrease in GLT-1 mRNA levels (Kim et al., 2018b). This decrease was not observed following ShA cocaine self-administration and extinction training, suggesting that these changes in NAc GLT-1 mRNA are unique to LgA cocaine self-administration and abstinence. Furthermore, preliminary results also showed that LgA cocaine self-administration and prolonged abstinence leads to a hypermethylation of the GLT-1 gene in the NAc, providing one rationale for the observed decrease in GLT-1 mRNA (Kim et al., 2018b).

Restored GLT-1 expression is required but not sufficient to attenuate cocaine-seeking

Previous studies have demonstrated that pharmacological upregulation of GLT-1 expression in the NAc can attenuate reinstatement to cocaine seeking (Fischer et al., 2013; Knackstedt et al., 2010; LaCrosse et al., 2017; Reissner et al., 2014; Reissner et al., 2015; Sepulveda-Orengo et al., 2018). In the case of ceftriaxone, N-acetylcysteine and propentofylline, all three pharmacotherapeutic interventions require the restored expression of both xCT and GLT-1, or GLT-1 specifically (LaCrosse et al., 2017; Reissner et al., 2014; Reissner et al., 2015). These experiments highlight the importance of restored NAc GLT-1 expression in decreasing cocaine seeking. However, these pharmacological compounds have numerous effects in addition to upregulating GLT-1. For example, ceftriaxone also increases xCT expression (Knackstedt et al., 2010), N-acetylcysteine increases the availability of cystine for cystine-glutamate exchange and increases antioxidant glutathione production (Nocito

Echevarria et al., 2017), and propentofylline acts as a phosphodiesterase inhibitor and adenosine antagonist, among other things (Sweitzer and De Leo, 2011). To better understand the role of GLT-1 in cocaine-seeking, the experiments outlined in Chapter 4 used an AAV to directly and selectively overexpress GLT-1 in the NAc.

Despite ample evidence which indicates that restored GLT-1 expression is required to attenuate cocaine seeking through pharmacological restoration of glutamate homeostasis, the results from experiment 4.2 show that AAV-induced upregulation of GLT-1 in the NAc had no effect on the incubation of cocaine craving (Fig 4.4a and 4.4b). This result is similar to a previous study which showed that AAV-induced upregulation of GLT-1 in the NAc had no effect on reinstatement following ShA cocaine self-administration and extinction training (Logan et al., 2018). These results collectively suggest that although restored GLT-1 expression is required for pharmacological compounds to attenuate cocaine reinstatement, restored GLT-1 expression alone is not sufficient to decrease cocaine seeking. In addition to glutamate uptake by GLT-1, other components of glutamate homeostasis including: basal extracellular glutamate levels, cystine-glutamate exchange, and the glutamatergic receptors have all been shown to play a vital role in cocaine-seeking (Kalivas, 2009; Kalivas et al., 2003; Scofield et al., 2016a; Scofield and Kalivas, 2014). Therefore, it is likely that pharmacological induced upregulation of GLT-1 expression likely works in conjunction with one or more of these factors to decrease cocaine seeking.

The lack of an effect of AAV-induced GLT-1 overexpression on cocaine seeking was evident despite a significant increase in synaptic colocalization. Results from experiment 4.1 showed that GLT-1-HA^{+} astrocytes exhibited a significant increase in synaptic colocalization in comparison to GLT-1-HA^{-} astrocytes (Fig 4.3a and 4.3b). Nevertheless, rats microinjected

with AAV-GfaABC1D-GLT1-HA displayed an incubation of cocaine craving, similarly as rats microinjected with a control virus (AAV-GfaABC1D-Lck-GFP) (Fig 4.4). These results imply that the modest but significant 14% increase in synaptic colocalization induced by GLT-1 overexpression was not sufficient to produce a behavioral response (Fig 5.1c). A previous study from our lab showed that following ShA cocaine self-administration, chronic ceftriaxone treatment during the last seven days of extinction training reversed the cocaine-induced decrease in synaptic colocalization (Scofield et al., 2016b). In this case, rats that were treated with ceftriaxone exhibited synaptic colocalization levels similar to rats that self-administered saline (Scofield et al., 2016b). Since ceftriaxone also increases expression of xCT, these results suggest that upregulating both GLT-1 and xCT levels in the NAc can enhance synaptic colocalization compared to upregulating GLT-1 alone. However, how GLT-1 induced an increase in synaptic colocalization remains unknown and is an interesting area for future research.

Summary of unanswered questions and future directions: Functional consequences of cocaine-induced changes in structural properties and synaptic colocalization of astrocytes

The results from the experiments outlined in this dissertation provide insights into how prolonged abstinence from LgA cocaine self-administration affects NAc astrocytes. Results from Chapter 3 indicate that in male rats, NAc astrocytes exist in an atrophic, retracted state following abstinence from LgA cocaine self-administration. However, *how* these changes occur remains unknown and an important area for future research. The results from Chapter 4 suggest that GLT-1 expression is likely not a mechanism for the retracted phenotype. Therefore, future studies can investigate other potential mechanisms that may lead to the decrease in surface area, volume and synaptic colocalization following abstinence from LgA

cocaine self-administration. As mentioned in Chapter 3, one possibility is stress-induced activation of glucocorticoid receptors. To understand how stress levels change as a response to prolonged abstinence from LgA cocaine self-administration, a future experiment can examine corticosterone levels at different timepoints during abstinence. To directly examine the relationship between glucocorticoid receptors and the cocaine-induced changes in NAc astrocytes, future experiments can examine how agonists and antagonists at the glucocorticoid receptor affect astrocyte morphology.

Another possibility for the observed changes in NAc astrocytes is cocaine-induced changes in actin/cytoskeletal dynamics of astrocytes. Numerous studies have shown that withdrawal from cocaine exposure results in changes to dendritic spines (Anderson and Self, 2017; Barrientos et al., 2018; Christian et al., 2017; DePoy et al., 2014; Dumitriu et al., 2012; Shen et al., 2009). Other studies have shown that cocaine influences rho-associated protein kinase (ROCK) signaling pathways, which can potentially lead to these cytoskeletal changes (Kim et al., 2009; Swanson et al., 2017). However, it is unclear if these changes are exclusive to neuronal morphology, or if changes are also associated with alterations in astrocyte morphology. More recently, studies have implicated rho GTPases such as RhoA and Rac1 in regulating astrocyte morphology (Zeug et al., 2018; Zhang et al., 2019b). However, it remains to be studied if abstinence from LgA cocaine self-administration affects either small GTPases or ROCK signaling, and how these changes might influence astrocyte morphology.

The results from Chapter 4 suggest that the cocaine-induced changes in astrocyte morphology are independent of the decrease in synaptic colocalization. Therefore, future studies can examine how abstinence from LgA cocaine self-administration leads to this decrease in synaptic colocalization. Likewise, another interesting area for future research is to

determine how upregulating GLT-1 enhances synaptic colocalization, and importantly how to enhance this effect. One possibility is to examine how astrocytic synaptogenic factors and adhesion molecules change as a consequence of cocaine exposure, and how GLT-1 is involved in these changes. As discussed in Chapter 4, one candidate is the gap junction protein Cx30. Another interesting candidate is astrocytic neuroligins, which play a vital role in the morphogenesis and synaptogenesis of astrocytes (Stogsdill et al., 2017). Furthermore, astrocyte-derived adhesion molecules play an important role in astrocyte migration (Cardenas et al., 2014; Liddelow and Hoyer, 2016). However, how astrocytic synaptogenic proteins and adhesion molecules are influenced by cocaine, and further, how they are related to synaptic colocalization of astrocytes remains to be examined in greater detail.

The results from Chapter 4 also show that the modest 14% increase in synaptic colocalization induced by GLT-1 overexpression was not enough to attenuate cocaine seeking. Of relevance, the decrease in synaptic colocalization observed following LgA cocaine self-administration and abstinence in male rats is 34% (Fig 3.3d). These results raise the question if perhaps enhanced stimulation of NAc astrocytes is needed to augment synaptic colocalization and/or oppose cocaine seeking (Fig 5.1d). One way to investigate this question is to use astrocyte specific DREADDs to increase intracellular calcium signaling (Bang et al., 2016; Bonder and McCarthy, 2014). One hypothesis is that heightened astrocytic calcium signaling may lead to changes in actin/cytoskeletal dynamics of astrocytes, which may prevent or even reverse the cocaine-induced decrease in the morphometric properties and synaptic colocalization of NAc astrocytes. In support of this hypothesis, astrocyte specific Gq DREADDs decrease reinstatement following ShA cocaine self-administration and extinction training, an effect mediated by an increase in glutamate release (Scofield et al., 2015).

Therefore, an interesting area for future study is to examine if astrocyte specific DREADDs influence the cocaine-induced changes in NAc astrocytes via modulating actin/cytoskeletal dynamics of astrocytes (Fig 5.1d).

The findings from this dissertation show that these changes in astrocytes are only observed in male rats. Abstinence from LgA cocaine self-administration had no effect on astrocytes from the NAc of female rats, and further, had no effect on NAc GLT-1 expression in female rats. These findings raise important questions regarding what mechanisms and signaling pathways drive cocaine seeking in female rats. As discussed in Chapters 2 and 3, I observed no correlation between estrous cycle stage and cocaine-induced changes in NAc GLT-1 expression or the cocaine-induced changes in NAc astrocytes. However, since the estrous cycle was only noted following the 45-day abstinence period, future experiments can monitor the estrous cycle throughout self-administration, as well as throughout the duration of abstinence. Furthermore, to directly examine the contribution of sex hormones to the changes in NAc GLT-1 expression, as well as the changes in NAc astrocytes following abstinence from LgA cocaine self-administration, future experiments can utilize ovariectomized female rats. By supplementing ovariectomized female rats with sex hormones such as estrogen, progesterone or tamoxifen, the effects of these hormones on the cocaine-induced changes in GLT-1 expression, as well as NAc astrocytes can then be directly examined. Furthermore, it is currently unclear *how* these sex hormones exert their neuroprotective effects. The signaling pathways and mechanisms that contribute to this phenomenon remains to be discovered and is an important area for future research.

The findings presented in this dissertation characterize the structural adaptations of NAc astrocytes following abstinence from LgA cocaine self-administration. Furthermore, the

results described in this dissertation examine how these structural changes may be related to changes in NAc GLT-1 expression. However, in addition to glutamate uptake via GLT-1, astrocytes subserve a wealth of functions within the central nervous system, including: providing metabolic support to neurons, maintaining neuronal homeostasis, regulating blood flow, transmitter uptake and release, synapse development and synaptic signaling (Allen and Barres, 2009; Sofroniew and Vinters, 2010). It is currently unclear how the structural changes in astrocytes are related to these functions. Currently, it can only be hypothesized that the atrophy of astrocytes following abstinence from LgA cocaine self-administration also leads to a maladaptive physiological response. Therefore, one avenue for future research is to examine the relationship between astrocyte structure and function. One way in which this can be accomplished is by manipulating astrocyte activity. Previous studies have demonstrated the efficacy of both optogenetic (Akerboom et al., 2013; Ji and Wang, 2015; Perea et al., 2014) and chemogenetic (Bang et al., 2016; Bonder and McCarthy, 2014; Scofield et al., 2015) approaches to manipulating various astrocyte functions. Moreover, chemogenetic activation of astrocytes has been shown to reduce both cocaine-seeking (Fig 5.1d) (Scofield et al., 2015) and motivation to self-administer ethanol (Bull et al., 2014). Future studies can use these techniques to examine how manipulating astrocyte activity affects astrocyte structure. One hypothesis that can be examined is if stimulating astrocyte activity following abstinence from LgA cocaine self-administration can prevent or even reverse the observed structural changes in astrocytes (Fig 5.1d). In addition to manipulating astrocyte activity, an alternative way to examine the association between astrocyte structure and function is to use genetically encoded calcium sensors to monitor astrocytic calcium signaling. Intracellular calcium signaling by astrocytes has been hypothesized to reflect astrocyte activity (Bindocci et al., 2017; Shigetomi

et al., 2016). Future studies can investigate the correlation between the structural adaptations of astrocytes and the changes in astrocytic calcium signaling following abstinence from LgA cocaine self-administration.

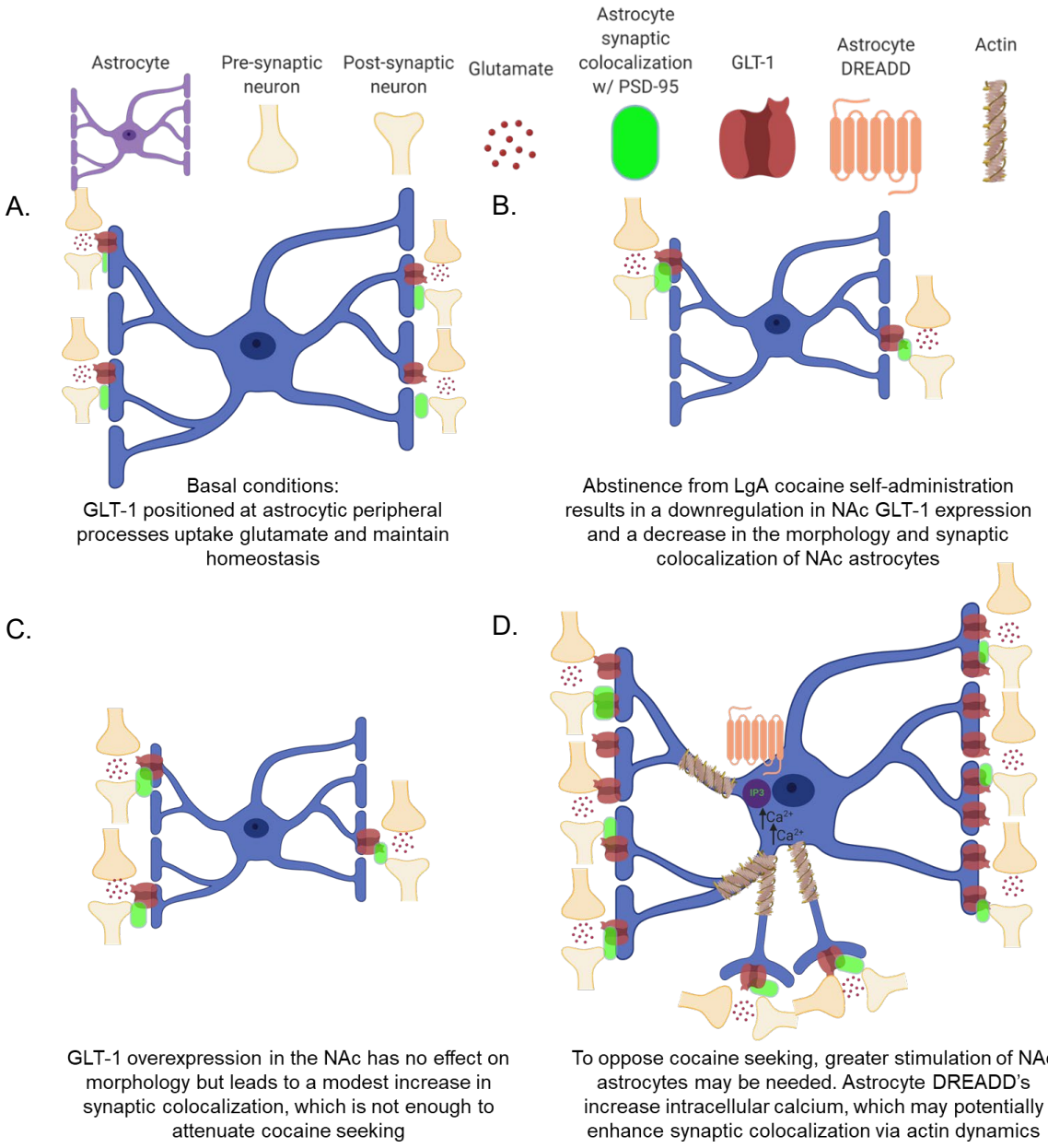

Figure 5.1 Proposed model of how enhanced stimulation of NAc astrocytes may oppose cocaine seeking. (A) At basal conditions, GLT-1 maintains glutamate homeostasis. **(B)** Following abstinence, NAc GLT-1 is downregulated, and NAc astrocytes exhibit a decrease in surface area, volume and synaptic colocalization. **(C)** AAV-induced GLT-1 overexpression in the NAc induces a modest increase in synaptic colocalization, but does not attenuate cocaine seeking. **(D)** Astrocyte specific DREADD's increase intracellular calcium and decrease cocaine and alcohol seeking (Scofield et al, 2015; Bull et al, 2014). It remains to be studied if enhanced stimulation of NAc astrocytes (via opto or chemogenetics) augments synaptic colocalization, and if this increase in colocalization opposes cocaine seeking.

Concluding Remarks

The results from this dissertation revealed sex differences in the structural adaptations of NAc astrocytes following prolonged abstinence from LgA cocaine self-administration. Whereas a significant decrease in surface area, volume, and synaptic colocalization of NAc astrocytes was found in male rats, this phenomenon was not observed in female rats. Likewise, although a significant downregulation in NAc GLT-1 expression was observed in male rats, this decrease was not found in female rats. The findings from this dissertation also showed that the relationship between NAc GLT-1 expression and the cocaine-induced decrease in the morphometric changes NAc astrocytes is correlational and not mechanistically linked. Furthermore, the downregulation in NAc GLT-1 is not a mechanism for the structural atrophy of astrocytes, but likely occurs either as a consequence of the structural changes in NAc astrocytes, or in parallel by an independent mechanism. Lastly, although restored GLT-1 expression is required for pharmacological compounds to attenuate cocaine-seeking, directly and selectively upregulating GLT-1 in the NAc does not affect cocaine-seeking following prolonged abstinence. Together, these data demonstrate that in male but not female rats, the structural changes in NAc astrocytes following abstinence from LgA cocaine self-administration may drive drug seeking following a period of abstinence. However, these changes in the morphometric properties and synaptic colocalization in NAc astrocytes is independent of GLT-1 expression. Future experiments can investigate a potential mechanism for these structural changes in astrocytes in male rats, as well as whether stimulation of synaptic colocalization of astrocytes processes can inhibit incubation of cocaine craving. Lastly, future studies will be critical to investigate the mechanism(s) driving sex differences in the structural

adaptations and synaptic colocalization of astrocytes following abstinence from LgA cocaine self-administration.

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